Characterization of Non-*aureus* Staphylococci Isolated from Intramammary Infections and Extramammary Sites on Dairy Farms

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ABSTRACT

This thesis is an investigation of the diagnosis, udder health effects and sources of non-*aureus* staphylococcal (NAS) intramammary infections (IMI) on dairy farms. The first objective of this series of studies was to evaluate the diagnostic accuracy of different target genes (*rpoB* or *cpn60*) and reference databases (GenBank or Chaperonin) to identify NAS species. The use of both *rpoB* and *cpn60* genes using either databases provided similar accuracy in the identification of NAS species, although the highest proportion of NAS species was correctly identified with the *rpoB* gene using the GenBank database.

The second objective was to determine the effects of NAS species on udder health and milk yield compared with healthy quarters and quarters infected with a major pathogen. The majority of NAS species had higher somatic cell counts (SCC) than healthy quarters and lower SCC than *S. aureus*-positive quarters. These SCC values were within the range of what is considered a 'healthy' SCC level. There was no effect of NAS IMI on overall milk yield.

The third objective was to characterize the distribution of NAS isolated from the environment, body sites and IMI on dairy facilities and to determine potential sources of NAS IMI by comparing isolates from milk samples and extramammary sites. *Staphylococcus chromogenes* was identified exclusively from milk samples and represented the largest proportion of isolates. *Staphylococcus equorum* was identified exclusively from body and environmental site samples. *Staphylococcus xylosus* was the only NAS species that was identified from all sample types and was present in both milk and extramammary sites from the same cow.

The fourth set of objectives were to determine (1) the proportion of specific *S. chromogenes* genotypes on dairy farms; (2) the effect of these genotypes on SCC; and (3) the persistency of each genotype within quarters. Of all 4 *S. chromogenes* genotypes, Genotype 1 was the most predominant in the study and had the largest proportion of persistent IMI identified. There was no effect of SCC among all genotypes and SCC levels were considered to be healthy.

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DEDICATION

I dedicate this thesis to my daughter, Eleanor, who has brought such love and laughter into my life. You never fail to put a smile on my face and made this writing process a little more bearable. You are the ultimate inspiration I need to achieve my goals and have put life into a whole new perspective. I love you more than words can say.

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LIST OF ABBREVIATIONS

AFLP	amplification fragment length polymorphism
BMSCC	bulk milk somatic cell count
CBMMQRN MPCC	Canadian Bovine Mastitis and Milk Quality Research Network Mastitis Pathogen Culture Collection
CI	confidence interval
DIM	days in milk
DNA	deoxyribonucleic acid
HRMA	high-resolution melt analysis
IMI	intramammary infection
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
NCDF	National Cohort of Dairy Farms
NAS	non-*aureus* staphylococci
NMC	National Mastitis Council
NPV	negative predictive value
OR	odds ratio
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PPV	positive predictive value
SCC	somatic cell count
SCS	somatic cell score
SE	standard error
Se	sensitivity
Sp	specificity
TSB	tryptic soy broth
WGS	whole-genome sequencing

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Mastitis continues to be an extremely detrimental disease to the dairy industry. In Canada alone, the dairy industry suffers economic losses of up to 400 million CAD each year (CBMMQRN, 2010), with 31% of all lactating dairy cattle in Canada experiencing a case of clinical mastitis annually (CBMMQRN, 2009). In western Canada, impaired udder health, defined as either mastitis or high SCC, was the second most common reason for culling in dairy herds during 2017 (CanWest DHI, 2018). As mastitis can have a devastating impact on dairy cattle and producers, it is important that research continues in this field so dairy producers, scientists and veterinarians can better understand and control this disease.

As non-*aureus* staphylococcal (NAS) IMI are extremely prevalent, it is important to understand how this group of pathogens affects dairy cattle. This literature review will discuss the ecology of NAS in dairy cattle, identification and treatment methods, as well as the impact that this group of pathogens has on dairy cattle.

1.2 PROPERTIES OF NON-*AUREUS* STAPHYLOCOCCI

There have been over 50 NAS species and subspecies identified (Pyörälä and Taponen, 2009). Although NAS have historically been considered as apathogenic when isolated from the udder, NAS have been shown to have varying virulence and persistency and it has been demonstrated that this group of pathogens can have detrimental effects on both human and animal health (Huebner and Goldmann, 1999).

Non-*aureus* staphylococci have previously been classified as contagious or host-adapted pathogens (Blowey and Edmondson, 2010). However, current research suggests that some NAS species can behave as environmental pathogens (Piessens et al., 2012b). These organisms are commonly isolated from the teat skin, teat end and teat canal (Blowey and Edmondson, 2010). Non-*aureus* staphylococci can be isolated from teat apices in cows that do not display any clinical signs of IMI (Braem et al., 2013). It has been shown that for many NAS species,

especially *Staphylococcus chromogenes* (*S. chromogenes*), the pathogen can be isolated from the teat canal approximately 1-2 wk before an IMI with the same NAS species (Quirk et al., 2012). These pathogens are associated with subclinical and clinical IMI and have been isolated from lactating cattle, as well as maiden and pregnant heifers (Blowey and Edmondson, 2010). Heifers can become infected with NAS prior to parturition and the IMI can continue following calving, although prevalence tends to decline as lactation progresses (Matthews et al., 1992; Krömker and Friedrich, 2009). Although NAS have been recovered from both clinical and subclinical cases of mastitis, they are not a common cause of clinical mastitis, with approximately 5.1% (Olde Riekerink et al., 2008) of clinical mastitis cases being due to NAS.

Evidence varies as to how NAS interacts with other mastitis pathogens. Some evidence suggests that NAS IMI can protect against IMI by major pathogens, such as *Escherichia coli* (*E. coli*; Bradley, 2002). In addition, cows that have both *S. aureus* and NAS IMI in the same quarter prior to dry-off have a 5.1 increased odds ($P = 0.08$) of curing the *S. aureus* IMI compared with cows that did not have NAS IMI present in the same quarter (Dingwell et al., 2003). However, these results may not be similar with all major pathogens, as a different study demonstrated that cows with a previous NAS IMI had a lower prevalence of *S. aureus*, but a higher prevalence of *Streptococcus agalactiae* (*Strep. agalactiae*) than previously uninfected cows (Nickerson and Boddie, 1994). In contrast to these results, a recent study found that previous NAS IMI is a risk factor for new *S. aureus* and *E. coli* IMI and a similar relationship was seen with *Strep. uberis* (Reyher et al., 2012a). The discrepancies among these studies could in part be due to the fact that NAS was classified as a single pathogen, when in reality, each NAS species will interact differently with each major pathogen. In addition, the type of study conducted could alter the understanding of what protective roles NAS species have in the udder when comparing challenge and naturally-occurring IMI (Reyher et al., 2012b). Nevertheless, it is apparent that major pathogens interact differently with NAS and it is important to understand how individual NAS species affect the mammary gland of dairy cattle.

1.3 IMPACTS OF NON-*AUREUS* STAPHYLOCOCCAL INTRAMAMMARY INFECTIONS

1.3.1 Impacts on Milk Yield

There are many possible impacts of NAS IMI, with cost and welfare having the most potential. It is estimated that cows infected with NAS throughout a single lactation will have a decrease in mature equivalent lactation milk yield of 821 kg compared to uninfected cows (Timms and Schultz, 1987). However, recent research has shown contradictory results. Heifers that freshen with NAS IMI can produce an extra 2.9 kg/d of milk during their first lactation in comparison to their uninfected counterparts (Piepers et al., 2010). This study also found that these heifers had a lower incidence of clinical mastitis throughout their first lactation than uninfected heifers (3.6 and 21%, respectively). A follow-up study found that heifers with NAS IMI produce an extra 2 kg/d of milk than uninfected animals, even when accounting for confounding factors such as herd, genetic merit for milk yield, 305-d milk yield of a herd, clinical mastitis cases occurring in the lactation and test day SCC (Piepers et al., 2013). These animals also tended to have lower incidence of clinical mastitis throughout their first lactation than uninfected heifers. Another study supports this finding, where cows infected with NAS throughout their lactation produced 0.5 kg/d more milk compared to culture-negative cows (Schukken et al., 2009).

Contradictory results were found in a twin heifer study in which the twin that freshened with NAS IMI did not have significantly different milk yield over their first 200 DIM compared with their uninfected twin (Pearson et al., 2013). Similar findings were found in a study that compared both milk yield and milk components between quarters diagnosed with NAS IMI and uninfected contralateral quarters (Tomazi et al., 2015). The study found that there was no difference in milk yield or components between the infected and uninfected quarters.

1.3.2 Impacts on Udder Health

In addition to effects on milk yield, NAS IMI have been associated with increased quarter milk SCC (Sampimon et al., 2009b), composite milk SCC (Gillespie et al., 2009) and bulk milk somatic cell count (BMSCC; Blowey and Edmondson, 2010). In one study, quarters that were

infected with NAS had a geometric mean SCC of 109,000 cells/mL (Sampimon et al., 2009b). This is generally considered a moderate SCC level but was double that of uninfected quarters. It has been shown that animals, particularly heifers, that contracted a NAS IMI during the first month following freshening were at greater risk of having increased SCC for the remainder of their lactation compared with those animals that were uninfected in the month following calving (Paradis et al., 2010). Another study found that on a cow-level, animals infected with NAS had a higher linear score than uninfected animals (Schukken et al., 2009). In addition, authors found that NAS IMI contributed to a higher proportion of BMSCC (17.9%) than any major pathogen in herds with a BMSCC of <200,000 cells/mL and this proportion decreased as BMSCC increased. This is supported by another study that found cows with a NAS IMI that were categorized as having high SCC were more prevalent in herds with a BMSCC of <150,000 cells/mL than major pathogens and this prevalence also decreased as BMSCC increased (Sampimon et al., 2010). This suggests that NAS may have increased importance in low SCC herds.

Different NAS species can also have differing effects on SCC. One study found that *S. chromogenes*, *S. simulans* and *S. xylosus* were associated with increased SCC levels in infected quarters, which were comparable to quarters infected with *S. aureus* (Supré et al., 2011). Similarly, a study that determined mean SCC on Canadian dairies found that quarters with *S. chromogenes* and *S. simulans* had higher SCC than uninfected quarters (Fry et al., 2014). In this study, there were other NAS species that also had higher SCC levels than culture-negative quarters (*S. capitis*, *S. epidermidis*, *S. haemolyticus*, *S. hyicus*, *S. warneri* and *S. xylosus*), however they were not as prevalent or persistent as *S. chromogenes* and *S. simulans*, so they may not have as great an effect on udder health. A follow-up study reported that 65.5% of NAS isolates resulted in infections with low SCC levels (<200,000 cells/mL), with 50.4% of these represented by *S. chromogenes*, followed by *S. simulans* (15.5%) and *S. xylosus* (12.6%; Condas et al., 2017b). Of the high SCC quarters, 46.2% of isolates were represented by *S. chromogenes*, followed by *S. simulans* (19.2%) and *S. xylosus* (9.5%).

1.4 INCIDENCE AND PREVALENCE RATES OF NON-*AUREUS* STAPHYLOCOCCI

1.4.1 Incidence of Non-*aureus* Staphylococci

Between 2007 and 2008, it was estimated that the NAS incidence rate on Canadian dairy herds was 0.29 IMI per quarter-month (Dufour et al., 2012). In this study, the prevalence of NAS was 42.7% on a quarter-level. In another study that determined the incidence rate of clinical IMI for a variety of different pathogens, the incidence rate of clinical NAS IMI was quite low, at a rate of 0.0176 cases per cow-year at risk (Barkema et al., 1998). In comparison, the incidence rates of major pathogens, such as *Strep. dysgalactiae*, *S. aureus* and *E. coli*, were 0.0344, 0.0628 and 0.0527 cases per cow-year at risk, respectively. Another study that determined the incidence rate of clinical mastitis cases across Canadian dairy farms found that NAS was frequently isolated from clinical samples, however, only resulted in an incidence rate of 0.0115 cases per cow-year at risk (Olde Riekerink et al., 2008). It was speculated that NAS was not the primary causative agent in the majority of the clinical mastitis cases and was just present in the teat canal.

1.4.2 Prevalence of Non-*aureus* Staphylococci

As shown in previous literature, some pathogens tend to be more prevalent than others (Sampimon et al., 2009a). This is also true with NAS species. A study that examined a subset of isolates identified with NAS species from Canadian dairy herds found that *S. chromogenes* accounted for almost half the isolates (48%), followed by *S. simulans* (19%) and *S. xylosus* (10%) (Fry et al., 2014); all of which had the ability to cause persistent IMI. A follow-up study found similar results, where *S. chromogenes* accounted for 48.9% of NAS isolates, followed by *S. simulans* (16.8%) and *S. xylosus* (11.6%; Condas et al., 2017a)

One study found that on a quarter-level, *S. chromogenes* (48%) was the most prevalent species on 3 research herds in the United States followed by *S. hyicus* (26%), *S. epidermidis* (24%) and *S. simulans* (7%; Gillespie et al., 2009). When strain-typing, these authors found 33 different strains of *S. chromogenes*, 19 different strains of *S. hyicus*, 21 different strains of *S. epidermidis* and 5 different strains of *S. simulans*. None of these strains were present on all 3 study herds, indicating that NAS strains can be very diverse among farms. The authors also

consistently isolated particular strains from the same cow for periods of a week up to 10 mo, indicating that NAS IMI have varying and potentially long-term persistency in the udder. Persistency can vary among species, as it has been shown that *S. chromogenes* can cause more persistent than transient IMI in quarters, while *S. cohnii*, *S. haemolyticus* and *S. simulans* tend to cause more transient IMI and *S. fleurettii* solely causes transient IMI (Supré et al., 2011).

In a study that investigated the prevalence of IMI on Dutch dairy herds, NAS was commonly isolated from both high (≥250,000 cells/mL) and low (<150,000 cells/mL) SCC cows (14.6 and 10.4% at a quarter-level and 38.5 and 30.1% at a cow-level, respectively) and was present in all study herds (Sampimon et al., 2009a). There was a higher prevalence of NAS IMI in primiparous cows in comparison to multiparous cows. In fact, it has been shown that NAS have been isolated from streak canals of nulliparous heifers, ranging from 6-24 mo (White et al., 1989), which may explain the increased level of NAS found in primiparous animals after they freshen.

1.5 RISK FACTORS FOR NON-*AUREUS* STAPHYLOCOCCAL INTRAMAMMARY INFECTIONS

Although NAS have been shown to be extremely complex pathogens, there are identified risk factors for IMI. A study that characterized risk factors for NAS IMI was able to clearly identify management factors on dairy herds, which corresponded to NAS IMI incidence, prevalence and elimination (Dufour et al., 2012). Bedding and housing facilities played a key role in whether the cow acquired a NAS IMI. As compared with no outdoor access, cows that had access to pasture had lower odds (OR = 0.52) of acquiring an IMI. When comparing cows housed on straw bedding, cows bedded on sand or wood-based products had lower odds (OR = 0.27 and 0.55, respectively) of acquiring an IMI. In addition, cows in herds where employees received an incentive for good milk quality had lower odds (OR = 0.33) of becoming infected with NAS.

As compared with no outdoor access, prevalence was decreased in cows that had pasture-access (OR = 0.71), or were housed on sand or wood-based bedding (OR = 0.39 and 0.48, respectively) as compared to straw bedding. When comparing herds that did not dry teats during

milking, herds that used either paper or reusable cloth towels during milking (OR = 0.51 and 0.39, respectively) had a lower IMI prevalence. Lower prevalence was also demonstrated in herds where fresh cows were held in maternity pens for >7 d post-calving (OR = 0.12) and those that provided incentives to milkers for good milk quality (OR = 0.27; Dufour et al., 2012).

In contrast to NAS IMI incidence, there were many more risk factors associated with elimination rather than acquisition of IMI. Similar to IMI incidence, higher odds of eliminating IMI occurred for cows housed on sand bedding compared with those housed on straw (OR = 4.9). In the maternity pens, cows had lower odds of eliminating the IMI if the pens were bedded with straw (OR = 0.2) compared to wood products and bedded between once per day to once per month (OR = 0.37) as compared with bedding more than once per day. In addition, cows that had dirty lower legs had higher odds of IMI elimination (OR = 2.9) when compared to cows with very clean lower legs. Herds that monitored milk conductivity had lower odds of eliminating IMI (OR = 0.16). If herds had purchased cattle in the 6 mo prior to sampling, they had higher odds of eliminating IMI (OR = 2.3; Dufour et al., 2012) compared to closed herds.

When observing NAS species that behave as environmental pathogens, it has been shown that cows that spend greater than 2.5 h lying down following milking have a greater risk of acquiring a NAS IMI (DeVries et al., 2011). As it is evident that NAS IMI has been correlated with environmental management strategies (Dufour et al., 2012), it is not surprising that this correlation of increased exposure to bedding surfaces is associated with higher rates of NAS IMI. Similarly, another study found that dirty teat apices predisposed quarters to having IMI with NAS species deemed as 'environmental' (*S. cohnii*, *S. equorum*, *S. saprophyticus* and *S. sciuri*), however teat hygiene did not affect *S. chromogenes*; a species that appeared to be host-adapted (De Visscher et al., 2016a).

Along with environmental risk factors, it is important to look at other areas of IMI transmission, such as during milking. As with many types of pathogens, colonization of the skin from human hands has been shown to be a risk factor for IMI, therefore it has been suggested that good milking management practices should be observed and gloves should be worn during milking (Thorberg et al., 2006). Specifically, the same strains of *S. epidermidis* have been

isolated from both bovine milk and hand skin of producers on the same facility. In addition to *S. epidermidis*, *S. chromogenes* has been shown to have udder-adapted characteristics and cause a large number of IMI (De Vliegher, 2013). A study that looked at the risk factors of acquiring an IMI from either *S. chromogenes*, that was characterized as a 'host-adapted' species, or *S. chromogenes*, *S. simulans* and *S. xylosus*, that were characterized as 'relevant' species, found that inverted teat ends increased the chance of IMI from both of these categories of NAS pathogens (De Visscher et al., 2016a). As cows with inverted teat ends are predisposed to extensive milk leakage (Klaas et al., 2005), it important to ensure that proper milking procedures are adhered to so milk leakage is decreased before and after milking and pathogens have less exposure to teat ends.

1.5.1 Non-*aureus* Staphylococci Isolated from Extramammary Sites

To draw a direct link between NAS found in IMI and those potentially found in extramammary sites, isolating and identifying these pathogens has proven to be very informative in udder health research. Taponen et al. (2008) found that similar strains of *S. chromogenes*, *S. simulans* and *S. epidermidis* were shared between IMI and extramammary sites, however the majority of NAS species were separate between the 2 locations. Non-*aureus* staphylococci have also been characterized from extramammary sites in heifers; specifically from teat and inguinal skin (Adkins et al., 2018a). This study found that the most common NAS species isolated from extramammary sites were *S. haemolyticus*, *S. chromogenes* and *S. xylosus*, with the probability of *S. chromogenes* being isolated as a heifer's age increased. This may indicate that the risk of an animal acquiring a *S. chromogenes* IMI can occur earlier than their first lactation and that this may be a complex pathogen to manage on-farm.

1.6 DIAGNOSIS OF NON-*AUREUS* STAPHYLOCOCCAL INTRAMAMMARY INFECTIONS

As the majority of NAS IMI are subclinical, diagnosis of IMI is generally made based by milk culture. As described by Hogan et al. (1999), milk is plated on blood agar plates and visual characteristics of colonies are assessed after both 24 and 48 h. Colonies appear as large and smooth and can present in a variety of colours, including white, cream and yellow. Isolates can

also display varying degrees of hemolysis. Subsequent tests are also completed after visual identification to confirm that the colonies are in fact NAS. Colonies will test positive with a gram stain, positive with a catalase test and negative with a tube coagulase test. Bacteria are further characterized to determine specific species and strains of NAS.

Although some previous studies have treated NAS species as having very similar characteristics (Pyörälä and Taponen, 2009), in reality, each species may be unique; from where it is most commonly isolated from the cow and on-farm, to how it affects udder health. The most common species that have been isolated from persistent subclinical IMI are *S. chromogenes*, *S. epidermidis* and *S. simulans*, with *S. chromogenes* being most prevalent in primiparous cows and *S. epidermidis* being most prevalent in multiparous cows (Thorberg et al., 2009). Although these species are commonly isolated from milk, it has been shown that many variations in NAS ecology are present. For example, a study characterizing different species of NAS from both milk and the environment found that 13 different species were isolated from milk, while 18, 19, 15 and 19 species were isolated from air, slatted floors, sawdust cubicles and sawdust stock, respectively (Piessens et al., 2011). Many of these species overlapped among sample areas and the prevalence of these species within each sampling niche was extremely variable. In addition, some of the more prominent NAS species, including *S. chromogenes*, *S. epidermidis*, *S. simulans* and *S. haemolyticus*, have been found to be present in both milk and environmental samples (Piessens et al., 2012a). After subtyping, it was found that a number of *S. haemolyticus* genotypes were isolated from the environment, which could correspond to environmental IMI, whereas genotypes of *S. epidermidis* and *S. chromogenes* seem to commonly correspond to a more host-adapted IMI. Due to this variation among different species, it is important that the species of NAS are identified correctly to get a full understanding of their ecology.

1.6.1 Phenotypic Determination of Non-*aureus* Staphylococcal Species

Approximately 40 years ago, some of the original techniques for the identification of human NAS isolates were published (Kloos and Schleifer, 1975) and since, these techniques have been used and modified for udder health research. There are essentially 2 methods to identify NAS species that have been used in research; through either phenotypic or genotypic

classification (Zadoks and Watts, 2009). Phenotypic identification focuses on isolate traits such as growth characteristics, morphology, biochemical characteristics and antimicrobial resistance (Zadoks and Watts, 2009). This method is commonly used for diagnostic purposes and a variety of commercial biochemical test systems are currently available (Zadoks and Watts, 2009). Although phenotypic methods have shown to be effective in determining NAS species isolated from humans, there are concerns with the variability, typeability and accuracy of these strategies for bovine mammary isolates (Zadoks and Watts, 2009). To be specific, the variability of results are due to inconsistencies among NAS isolates of the same species, as well as human error, as results may be based on subjective interpretations of these tests. Typeability refers to the ability of a typing system to correctly type specific isolates. For example, the typeability of a system increases if the amount of tests in that system increases. Finally, accuracy refers to the ability of a particular test to identify the same correct NAS species (Zadoks and Watts, 2009). Different phenotypic isolation systems have a variable degree of agreement (ranging between 45 and 94%) for the correct identification of species, which may not lead to accurate determination of NAS IMI (Ruegg, 2009). In addition, these phenotypic methods were originally developed for human isolates and have varying success rates using bovine milk samples (Sampimon et al., 2009c). These concerns about the quality and validity of phenotypic methods are justifiable; therefore the results obtained from these methods could be questionable.

1.6.2 Genotypic Determination of Non-*aureus* Staphylococcal Species

Genotypic classification is based on the DNA sequence of a chosen gene or series of genes (Zadoks and Watts, 2009). The benefits of genotypic classification are the improved accuracy of identification and the ability to identify specific strains of NAS, which can be extremely valuable in understanding the ecology of each species to determine their specific impacts on udder health (Zadoks and Watts, 2009; Leroy et al., 2015). There are a variety of genotypic methods to identify species, which include both conventional PCR and real-time qPCR followed by DNA-sequencing (Zadoks and Watts, 2009), high-resolution melt analysis (Ajitkumar et al., 2013), amplified-fragment length polymorphism (Piessens et al., 2012a), pulsed-field gel electrophoresis (Mørk et al., 2012), random amplification of polymorphic DNA analysis (Piessens et al., 2012a), automated ribotyping (Carretto et al., 2005) and tRNA-

intergenic length polymorphism (Stepanović et al., 2005). In contrast to phenotypic methods, which questions the variability, typeability and accuracy of the tests, genotypic methods have high typeability and reproducibility and are highly discriminatory (Zadoks and Watts, 2009), which makes genotypic identification a superior choice for the identification of NAS species from bovine IMI.

One of the most common genes currently used in the identification of NAS species is 16S rRNA. Although 16S rRNA has been used successfully in research, it can be present in multiple copies in a genome (Větrovský and Baldrian, 2013) and is limited in its ability to distinguish among closely related NAS species (Zadoks and Watts, 2009). Another commonly used gene in udder health research, *rpoB*, is a frequent single-copy target gene successfully used for identifying NAS isolates from bovine milk (Zadoks and Watts, 2009; Case et al., 2007). As an alternative to 16S rRNA or *rpoB*, the single-copy *cpn60* gene provides a universal target that has been shown to be extremely discriminatory in providing informative data regarding the phylogenetic properties of similar species (Zadoks and Watts, 2009; Hill et al., 2004). This gene has been shown to be extremely accurate in identifying NAS species (Goh et al., 1996; Kwok et al., 1999), however has not been used extensively in udder health research. One of the key benefits of using the *cpn60* gene is the Chaperonin database, which is available online (Hill et al., 2004). This curated database is continually updated and currently contains over 25,000 searchable records to help identify the specific sequence in question. In addition, this database provides the percentage of similarity between reference sequences provided from the database and the NAS isolates in question, as well as visual structures of the sequences to identify any discrepancies that may be present.

1.6.3 Novel Species Determination Methods

A relatively new technology that can be used to identify specific organisms is whole-genome sequencing (WGS). This molecular technique uses the genetic information of organisms, much like the techniques listed above, but instead of targeting a single gene, it takes into account the entire genetic makeup of the pathogen in question. To the knowledge of the author, this technique has not been used extensively in udder health research to identify NAS species, yet it

has the potential to improve the understanding of this group of pathogens by allowing a better understanding of their phylogeny (Naushad et al., 2016). One of the limitations of WGS has been price, however, in the past 10 years the price of this technique has decrease substantially (NHGRI, 2016). Currently, the cost of WGS of a bacterial genome is approximately 100 CAD (Hasnain et al., 2015), making this a more realistic technique to use for molecular studies.

Another technique that has been used successfully in NAS identification from IMI is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Cameron et al., 2017). This approach studies the proteome of pathogens and allows for accurate species determination and high throughput of samples (Loonen et al., 2012). Different NAS species have been successfully identified using MALDI-TOF MS (Loonen et al., 2012) and udder health researchers have begun to use it as a method of species identification (Frey et al., 2013; Adkins et al., 2018b; Cameron et al., 2018).

1.6.4 Reclassification of Non-*aureus* Staphylococcal Species

With the increased number of studies focusing on NAS identification, as well as improved diagnostic techniques and reference databases, it has been suggested that some previously identified NAS species should be reclassified. An example of this is the reclassification of *S. pulvereri* to *S. vitulinus*. Past research has found that isolates identified as *S. vitulinus* and *S. pulvereri* were phylogenetically similar and that they may be the same NAS species, or that *S. pulvereri* may be a subspecies of *S. vitulinus* (Kwok and Chow, 2003; Petráš, 1998). As these species were so similar, the suggestion was made to reclassify *S. pulvereri* as a synonym of *S. vitulinus* (Švec et al., 2004) and many studies now identify both species as *S. vitulinus* (Supré et al., 2009). Another NAS species that has proven to be difficult to identify is *S. agnetis*. This species is phylogenetically similar to *S. hyicus* and *S. chromogenes* (Taponen et al., 2012) and it has been suggested that in previous research, *S. agnetis* isolates were misclassified as *S. hyicus* (Calcutt et al., 2014). Although this reclassification has been established, proper identification of this species is still a concern (Cameron et al., 2018).

1.7 CONTROL AND TREATMENT OF NON-*AUREUS* STAPHYLOCOCCAL INTRAMAMMARY INFECTIONS

When assessing proper treatment strategies for NAS IMI, it is important to first determine which management practices are currently present to see if they are beneficial or detrimental to udder health. One key suggestion to promote udder health is to apply post-milking teat dip as a disinfectant following milking. For the majority of NAS species, it has been found that the prevalence of NAS in cows that receive a post-milking teat dip decreased compared to cows that did not receive a post-milking teat dip (Quirk et al., 2012). Internal teat sealants can also be implemented into management programs to try to prevent NAS IMI during the dry period (Pyörälä and Taponen, 2009). However, as effective as these management strategies can be, there are times when antibiotic treatments are necessary.

Antibiotic treatment is a common solution to combat IMI. It has been demonstrated that antimicrobial treatments of NAS IMI have cure rates between 80 and 90% (Pyörälä and Taponen, 2009). One study showed that dry cow antibiotic treatment successfully eliminated NAS IMI in cows infected prior to dry-off as compared to untreated control cows (Harmon et al., 1986), however only penicillin-streptomycin was shown to be effective. In a study that compared 2 antibiotic treatments for treating clinical mastitis, penethamate hydriodide or a penicillin and dihydrostreptomycin combination, cows that received the penicillin and dihydrostreptomycin combination had a higher cure rate than those that received penethamate hydriodide (91.7 and 53.3%, respectively; McDougall, 1998). In this study, when subclinical IMI were assessed, there were no significant differences in cure rate between the 2 treatment groups (81.8 and 64.5%, respectively). Studies have examined the prepartum intramammary treatment of heifers, as these animals have been shown to be extremely susceptible to NAS IMI following calving. In heifers treated with pirlimycin hydrochloride prior to calving, it was found that those animals with a NAS IMI were more likely to cure after freshening compared to their untreated counterparts (Middleton et al., 2005). Although there was a benefit of cure rate due to treatment, there was no difference in milk yield between treated and untreated animals (Middleton et al., 2005). This could question whether treating heifers prepartum is an economically beneficial treatment strategy (Nickerson, 2009).

A large, comprehensive retrospective study that observed the efficacy of 7 different antibiotic treatments (amoxicillin, cephapirin, cloxacillin, erythromycin, hetacillin, penicillin or pirlimycin) on subclinical IMI from lactating dairy cows was completed using data taken throughout the years 1985 to 1996 from Quality Milk Promotion Services, located at Cornell University (Ithaca, NY, US; Wilson et al., 1999). Looking at NAS IMI specifically, it was found that there was no significant difference in cure rate between the non-treated cows and those in the antibiotic-treated group (72 and 81% cure rate, specifically). However, when looking at each specific antibiotic treatment separately, it was found that cows treated with amoxicillin had a significantly higher cure rate of NAS IMI (87% cure rate) than untreated cows. These results may suggest that there is value to treating even subclinical NAS IMI, provided that the correct antibiotic is selected for optimal cure rates.

Resistance of NAS IMI to antimicrobials has been a concern when considering appropriate treatment methods. A study testing the susceptibility of different antimicrobials to NAS IMI *in vitro* found that NAS were susceptible to ampicillin-subactam, cephalothin and gentamicin, and were resistant to erythromycin, oxacillin, penicillin and pirlimycin, with resistance to penicillin being the highest at 27.6% prevalence (Gentilini et al., 2002). As both erythromycin (Erythro-36, Vétoquinol, Lure, FR) and pirlimycin (Pirsue Sterile Solution, Zoetis, Florham Park, NJ, US) are commercially available as intramammary treatments for IMI in Canada, it is important to consider the resistance of NAS to these antimicrobials before treatment is administered. Looking at antimicrobial resistance from a large dataset in Canada, Nobrega et al. (2018) found that when treated systemically, NAS were also resistant to penicillin, as reported in the previous study, as well as third-generation cephalosporins and macrolides. There was no antimicrobial resistance associated with intramammary products, indicating that intramammary treatment of a NAS IMI can be a viable solution for producers. Recent work has characterized antimicrobial resistance among NAS species (Frey et al., 2013). Resistance varied from 2.4% of isolates (kanamycin and gentamicin) to 47% of isolates (oxacillin; Frey et al., 2013). Some NAS species from this study were found to be resistant to multiple antimicrobials and could therefore be difficult to treat, including *S. epidermidis*, *S. chromogenes* and *S. haemolyticus*. As NAS IMI has been frequently shown to cure spontaneously without antimicrobial treatment, there is merit

to weighing the benefits of treatment for each individual NAS IMI case (Pyörälä and Taponen, 2009).

Although treatment protocols are crucial to counteract IMI, it is just as important to implement preventative strategies. Vaccines have recently been studied to see if they are a viable option for producers (Middleton et al., 2009). Although NAS vaccination has shown some promise in research, Middleton et al. (2009) found that vaccinating cows with a *S. aureus* bacterin (Lysigin, Boehringer Ingelheim GmbH, Ingelheim am Rhein, DE) did not affect the risk of developing a new NAS IMI or SCC levels. This result was thought to be contributed to insufficient antibody levels available in the milk to aid in clearing NAS IMI. A combination vaccine composed of *S. aureus* and J5 *Escherichia coli* bacterins (Startvac, Hipra, Amer, ES) was also found to have no effect on the risk of developing a new NAS IMI, however, vaccinated animals had a higher cure rate than unvaccinated animals (Schukken et al., 2014). These authors did note that the efficacy of vaccine use is highly dependent on previously established management practices used to control IMI on-farm.

1.8 CONCLUSIONS

In conclusion, it is apparent that NAS are a very prevalent and complex group of pathogens. Although they are not as great of a concern as major pathogens, their effects can be potentially detrimental. This group of pathogens generally present themselves as chronic subclinical IMI and result in relatively low SCC levels. There are many risk factors that are associated with NAS IMI, which increases our understanding of this group of pathogens, as each species interacts with both the cow and the environment differently. As NAS are generally associated with subclinical cases of mastitis, diagnosis of these pathogens is generally carried out through culturing of milk samples. These NAS isolates can be further characterized using genotypic techniques, which can help identify how specific species affect the cow. To treat this pathogen group, preventative measures are the most successful techniques for control. Implementing post-dip in milking routines, applying teat sealant at dry-off and prepartum treatment of heifers can be beneficial when trying to decrease NAS IMI. Non-*aureus*

staphylococci are an interesting group of pathogens that should be further explored in research to get a better understanding of how each species affects both the udder and the cow.

1.9 RESEARCH OBJECTIVES

The first objective of this series of studies was to evaluate the diagnostic accuracy of different target genes (*rpoB* or *cpn60*) and reference databases (GenBank or Chaperonin) to identify NAS species. It was hypothesized that *cpn60* would be more discriminatory than *rpoB* for identifying NAS species isolated from milk.

The second objective was to determine the effects of NAS species on udder health and milk yield compared with healthy quarters and quarters infected with a major pathogen. It was hypothesized that NAS species as a group would have only minimal detrimental effects on udder health and milk yield compared to healthy quarters and that there would be distinct differences in udder health and milk yield among NAS groups.

The third objective was to characterize the distribution of NAS isolated from the environment, body sites and IMI on dairy facilities and to determine potential sources of NAS IMI by comparing isolates from milk samples with those from extramammary sites. It was hypothesized that there would be a within-cow or within-herd agreement for species that have either host-adapted or environmental behaviours.

The fourth set of objectives were to determine (1) the proportion of specific *S. chromogenes* genotypes on dairy farms; (2) the effect of these genotypes on SCC; and (3) the persistency of each genotype within quarters. It was hypothesized that there would be a predominant genotype identified from *S. chromogenes* isolates and that genotypes would yield persistent infections and increased SCC.

CHAPTER 2: IDENTIFICATION OF NON-*AUREUS* STAPHYLOCOCCI USING DIFFERENT TARGET GENES (*RPOB* OR *CPN60*) AND DATABASES (GENBANK OR CHAPERONIN)

This chapter evaluates the accuracy of identifying non-*aureus* staphylococcal (NAS) species using different target genes (*rpoB* or *cpn60*) and reference databases (GenBank or Chaperonin) compared with whole-genome sequencing (WGS). The use of both *rpoB* and *cpn60* genes using either the GenBank or Chaperonin databases provided similar accuracy in the identification of NAS species compared with WGS, although the highest proportion of NAS species were correctly identified with *rpoB* sequencing using the GenBank database.

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towards data analyses and manuscript writing. Luby was responsible for experimental design and intellectual contribution towards manuscript writing.

2.1 INTRODUCTION

Non-*aureus* staphylococci (NAS) are the most commonly isolated group of pathogens from IMI on dairy farms (Sampimon et al., 2009b). Although NAS are abundant on-farm, the majority of previous udder health research has focused on major pathogens, as they have the most detrimental effects on udder health. However, in the past decade or so, research groups around the world have conducted many studies to improve the understanding of the role NAS plays in udder health.

To date, over 50 species of NAS have been identified, many of which are found on dairy farms (Pyörälä and Taponen, 2009). Although there are a number of species that can cause IMI, NAS species have been classified together as a group. Milk culture has traditionally been the preferred method for diagnostic identification of NAS and is also used frequently in research. After plating on blood agar plates, the morphology, colour and pattern of hemolysis for each colony is considered when diagnosing NAS. Diagnosis is confirmed by gram stain, catalase and coagulase tube tests (Hogan et al., 1999). Although time consuming, with results being finalized 48 h after plating milk, this has been a very effective way to accurately identify NAS colonies.

For determining species of NAS isolates, both phenotypic and genotypic methods can be used (Zadoks and Watts, 2009). Originally developed for human isolates (Kloos and Schleifer, 1975), commercial phenotypic tests are available to identify NAS from bovine samples, such as the API Staph ID 32 and Staph-Zym tests (Sampimon et al., 2009c). Although convenient, these tests were only shown to correctly identify 41.3 and 30.8% of NAS species, respectively, compared with genotypic identification. There are a variety of genotypic methods that have been used to identify NAS; many of which focus on targeting specific genes of an organism (Zadoks and Watts, 2009). For NAS isolates of bovine mammary origin, 16S rRNA and *rpoB* genes are commonly used as targets (Cicconi-Hogan et al., 2014). It has been shown that the conserved 16S rRNA gene has its limitations when sequencing and discriminating among NAS species (Zadoks and Watts, 2009), as there can be multiple copies of this gene in the genome; potentially providing incorrect classification of isolates (Větrovský and Baldrian, 2013). However, the single-copy *rpoB* gene has the ability to provide similar, or even better, identification of closely

related species (Case et al., 2007). As an alternative to those target genes, the *cpn60* gene has been reported to be very accurate in identifying NAS species (Goh et al., 1996) and has also been shown to have similar or better identification than 16S rRNA (Schellenberg et al., 2009). To date, while *cpn60* has not been used extensively in udder health research, it has great potential in the identification of NAS.

In previous studies identifying NAS species, multiple target genes were selected to avoid the potential limitations of using only 1 target gene for species identification. Generally, the use of multiple target genes are done in series, whereby if a sequence is not identified with the primary target gene, the subsequent gene will be used for identification. Some of these gene combinations include 16S rRNA and *rpoB* (Park et al., 2011) 16S rRNA, *tuf* and *sodA* (Heikens et al., 2005) and 16S rRNA, *dnaJ*, *rpoB* and *tuf* (Lamers et al., 2012). A recent study performed whole-genome sequencing (WGS) on NAS isolates (Naushad et al., 2016). This comprehensive evaluation of NAS isolates greatly improves our knowledge of the similarities among species and provides an excellent reference with which to compare NAS sequences.

The objective of this study was to evaluate the diagnostic accuracy of different target genes (*rpoB* or *cpn60*) and reference databases (GenBank or Chaperonin) to identify NAS species. It was hypothesized that *cpn60* would be more discriminatory than *rpoB* for identifying NAS species isolated from milk.

2.2 MATERIALS AND METHODS

2.2.1 Sample Selection

Non-*aureus* staphylococcal isolates were acquired from the Canadian Bovine Mastitis and Milk Quality Research Network Mastitis Pathogen Culture Collection (CBMMQRN MPCC; Saint-Hyacinthe QC, CA), as described by Reyher et al. (2011). In brief, 91 herds from the National Cohort of Dairy Farms were enrolled across 6 Canadian provinces (Quebec, Ontario, Alberta, Prince Edward Island, New Brunswick and Nova Scotia) to participate in the project over a 2-yr period between 2007 and 2008.

Milk samples collected on these herds included samples from lactating dairy cows, both with and without clinical mastitis. Samples were processed in the laboratory for bacteriological culture using protocols established by the National Mastitis Council (Hogan et al., 1999). Based on these protocols, species that had significant growth on blood agar plates were selected to be stored in the CBMMQRN MPCC (Reyher et al., 2011).

A sample of 441 NAS isolates were selected for analysis from 5507 NAS isolates in the CBMMQRN MPCC, which represented 25 NAS species (Naushad et al., 2016). The distribution of these isolates amongst study provinces, herds and cows is described in detail by Naushad et al. (2016). The group of isolates included (Naushad et al., 2016): (1) clinical mastitis samples; (2) multi-drug resistance samples; (3) uncommon NAS species; and (4) only 1 common species isolated from each cow.

2.2.2 Whole-Genome Sequencing

Whole-genome sequencing of the 441 NAS isolates was conducted using procedures described by Naushad et al. (2016). In brief, DNA was extracted from isolates identified as NAS on blood agar plates and genome sequencing was performed. The retrieved sequences were then assembled and annotated, and the quality of these genome sequences were evaluated. The genome sequencing and assembly data were submitted to the National Center for Biotechnology Information under BioProject accession no. PRJNA342349.

2.2.3 Sequence Analysis

Sections of each whole-genome sequence were identified based on 2 target genes (*rpoB* or *cpn60*). These untrimmed sequences were processed for alignment and trimmed using reference primers with sequence analysis software (MEGA version 6.0, Center for Evolutionary Medicine and Informatics, Biodesign Institute, Arizona State University, Tempe, AZ, US; Tamura et al., 2013). The *rpoB* sequences were trimmed to a final sequence length of 556 bp based on reference primers 2643F (5'-CAATTCATGGACCAAGC-3') and 3241R (5'-GCIACI TGITCCATACCTGT-3'; Drancourt and Raoult, 2002). The *cpn60* sequences were trimmed to a

final sequence length of 552 bp based on reference primers H279 (5'-GAIIIIGCIGGIGAYGGIACIACIAC-3') and H280 (5'- YKIYKITCICCRAAICCIGGIGCYTT-3'; Goh et al., 1996).

All trimmed sequences were subjected to a BLAST search (Johnson et al., 2008) on the GenBank database (National Center for Biotechnology Information, Bethesda, MD, US; http://www.ncbi.nlm.nih.gov/genbank; Benson et al., 2013) and compared with reference nucleotide sequences to confirm NAS species identification. In addition, *cpn60* sequences were also subjected to a FASTA search (Pearson and Lipman, 1988) on the Chaperonin database (Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, CA; http://www.cpndb.ca; Hill et al., 2004) and compared with reference nucleotide sequences to confirm NAS species identification. Identity was confirmed if sequences had ≥97% homology with database results (Drancourt and Raoult, 2002), otherwise, sequences were categorized as 'unidentified'. Sequences were categorized as 'misidentified' if they had ≥97% homology with database results and were incorrectly identified or identified as multiple NAS species.

2.2.4 Data Analyses

As the 25 identified NAS species were represented by a varying number of sequences, descriptive data analysis was conducted on 5 previously established clades, as described by Naushad et al. (2016). These clades were established based on phylogenetic analysis of the 441 selected NAS sequences. The distribution of the number of sequences and species within each clade is described in Table 2.1.

Data analyses were modeled after a manuscript evaluating the use of 2 phenotypic tests for the identification of NAS (Sampimon et al., 2009c). Species included in the analyses represented ≥25 sequences from the selected NAS isolates. Four diagnostic accuracy measures [sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV)], as well as their corresponding 95% confidence intervals (CI), were used to evaluate the efficacy of both target genes and reference databases within each of the selected species.

Diagnostic accuracy measures were performed with statistical software (SAS version 9.4, SAS Institute Inc., Cary, NC) using frequency tables and statistics (PROC FREQ). Identification using target genes and reference databases were compared with identification using WGS, which was considered the 'gold standard' test.

Using *S. xylosus* as an example, the 4 diagnostic accuracy measures were defined as: (1) Se = number of samples (n) of *S. xylosus* isolates correctly identified with both WGS and individual gene sequencing / n of *S. xylosus* isolates correctly identified with WGS; (2) Sp = n of isolates other than *S. xylosus* correctly identified with both WGS and individual gene sequencing / n of isolates other than *S. xylosus* isolates correctly identified with WGS; (3) PPV = n of *S. xylosus* isolates correctly identified with both WGS and individual gene sequencing / n of *S. xylosus* isolates correctly identified with individual gene sequencing; and (4) NPV = n of isolates other than *S. xylosus* correctly identified with both WGS and individual gene sequencing / n of isolates other than *S. xylosus* correctly identified with individual gene sequencing.

2.3 RESULTS

There were 440 NAS sequences included for analyses after 1 sequence was removed due to incomplete sequence data. This sequence represented *Staphylococcus sciuri* (*S. sciuri*) from Clade A.

2.3.1 Descriptive Data

Using the GenBank database, the *rpoB* gene correctly identified 87.95% (n = 387; 95% CI= 84.57 - 90.68%) of all NAS sequences. Among clades, the proportion of correctly identified NAS sequences ranged from 81.56% (Clade E) to 100.00% (Clade C). The distribution and identification of NAS species and phylogenetic clades using the *rpoB* gene and GenBank database is reported in Table 2.2.

Using the GenBank database, the *cpn60* gene correctly identified 84.55% (n = 372; 95% CI= 80.86 - 87.63%) of all NAS sequences. Among clades, the proportion of correctly identified NAS sequences ranged from 73.74% (Clade B) to 97.16% (Clade E). The distribution and

identification of NAS species and phylogenetic clades using the *cpn60* gene and GenBank database is reported in Table 2.3.

Using the Chaperonin database, the *cpn60* gene correctly identified 87.05% (n = 383; 95% CI= 83.57 - 89.87%) of all NAS sequences. Among clades, the proportion of correctly identified NAS sequences ranged from 73.74% (Clade B) to 97.16% (Clade E). The distribution and identification of NAS species and phylogenetic clades using the *cpn60* gene and Chaperonin database is reported in Table 2.4.

2.3.2 Diagnostic Accuracy Measures

Diagnostic accuracy measures of 6 NAS species for all database and target gene combinations are reported in Table 2.5. Specificity and PPV for all species were 100.00%; therefore, only Se and NPV are reported in Table 2.5. Using the GenBank database and *rpoB* gene combination, all species had a Se and NPV of 100.00%, except for *S. epidermidis*, which had a Se of 96.15% (95% CI= 80.36 - 99.90%) and a NPV of 99.76% (95% CI= 98.66 - 99.99%). The Se for *S. epidermidis* using the GenBank database and *cpn60* gene combination and the Chaperonin database and *cpn60* gene combination was 3.85% (95% CI= 0.10 - 19.64%) and 100.00% (95% CI= 86.77 - 100.00%), respectively, with NPV of 94.31% (95% CI= 91.71 - 96.28%) and 100.00% (95% CI= 99.11 - 100.00%), respectively. Using *cpn60*, Se and NPV for *S. sciuri* was 100.00% for both databases. For the remaining NAS species, *cpn60* had identical Se and NPV using both databases, but were lower than Se and NPV found with the GenBank database and *rpoB* gene combination.

2.4 DISCUSSION

This study was conducted to evaluate the accuracy of identifying NAS species using different target genes and reference databases. It was hypothesized that *cpn60* would be more discriminatory than *rpoB* for identifying NAS species isolated from milk. To accurately characterize the role of NAS in udder health, it is important to select the most effective identification strategy to obtain the most accurate results.

The results of this study indicate that the use of both *rpoB* and *cpn60* genes using either the GenBank or Chaperonin databases provided similar accuracy in the identification of NAS species compared with WGS. Overall, the GenBank database using the *rpoB* gene correctly identified the greatest number of sequences, followed by the Chaperonin database using the *cpn60* gene and the GenBank database using the *cpn60* gene. Both *rpoB* (Case et al., 2007) and *cpn60* (Hill et al., 2004) are single-copy genes and are found only once in the genome, unlike genes such as 16S rRNA that can have multiple copies present in a single genome (Větrovský and Baldrian, 2013). For NAS identification, using single-copy genes as targets are very beneficial for accuracy, as it is certain that there will be no discrepancies in the gene section targeted. This is especially true when using this identification method for diagnostic purposes. Based on this characteristic of both *cpn60* and *rpoB* genes, it is not surprising that all database and gene combinations provided similar positive identification of sequences.

All database and gene combinations yielded 100% Sp and PPV for all selected NAS species. This indicates that there were no false positives for any combinations. Of the sequences that were incorrectly identified with various database and gene combinations, which were considered false negatives, a large number were unidentified with low homology compared with database results. In many studies, this issue has been avoided by using multiple target genes to identify sequences if one is below the pre-established homology threshold (Adkins et al., 2018b; Park et al., 2011). This can be a limitation of analyzing sequences with single genes (Lamers et al., 2012), depending on which species is in question. Of the remaining false negative sequences, these sequences were misidentified for another species, or had multiple species with the highest homology compared with the database. When looking at the full dataset, a high proportion of incorrectly identified sequences were misidentified, as opposed to unidentified, using *rpoB* for analysis (98.1%; n = 52) compared with both *cpn60* using the GenBank database (67.7%; n = 46) and *cpn60* using the Chaperonin database (47.4%; n = 27). As *cpn60* has been shown to be highly discriminatory among closely related NAS species, this could explain the lower rate of misidentified sequences compared with *rpoB* sequencing (Zadoks and Watts, 2009).

Among all of the database and target gene combinations, identification of 2 NAS species was incorrect: *S. agnetis* and *S. vitulinus*. Evaluation of *S. agnetis* has been reported to be a

challenge in recent studies and is commonly identified as *S. hyicus* (Cameron et al., 2018; Pizauro et al., 2017). This species was first reported in 2012 and it was found to be phylogenetically similar to both *S. chromogenes* and *S. hyicus* (Taponen et al., 2012), which are all represented in Clade B in the current study. As it is hypothesized that many NAS sequences that were previously identified as *S. hyicus* were actually *S. agnetis* (Calcutt et al., 2014), further research should be done in this field to increase the number of *S. agnetis* sequences that are available on reference databases. The other species that was incorrectly identified by all database and target gene combinations, *S. vitulinus*, was either identified as *S. pulvereri*, or had identical homology between *S. vitulinus* and *S. pulvereri*. Previous research has indicated a close phylogenetic similarity between *S. vitulinus* and *S. pulvereri*, and has suggested that *S. pulvereri* is in fact *S. vitulinus*, or a subspecies of this NAS species (Kwok and Chow, 2003; Petráš, 1998). In 2004, after re-evaluating reference strains, it was proposed that *S. pulvereri* should be reclassified as a synonym of *S. vitulinus* (Švec et al., 2004) and now is identified as such in NAS studies (Piessens et al., 2011; Supré et al., 2009). Re-evaluation of *S. pulvereri* sequences on reference databases should be considered to improve identification accuracy.

One limitation found in the current study was the incorrect identification of *S. devriesei* when using *cpn60* and the Chaperonin database. Upon further investigation, it was found that there were no records of *S. devriesei* in the Chaperonin database. The NAS sequences that were identified as *S. devriesei* with WGS were identified as *S. petrasii* with *cpn60* using the Chaperonin database and reference strains were sourced from human clinical isolates. *Staphylococcus devriesei* has been identified in udder health studies (Frey et al., 2013; Lange et al., 2015; De Visscher et al., 2016), however, *S. petrasii* is not described. Although absent from the Chaperonin database, it has been shown that *S. devriesei* has a relatively low prevalence in IMI, ranging between 0.5% (Lange et al., 2015) and 4.3% (Frey et al., 2013), therefore this may not significantly impair identification of NAS. The use of *cpn60* in udder health work is still not common, therefore the amount of sequences submitted to the GenBank database and subsequently entered into the Chaperonin database are limited.

2.5 CONCLUSIONS

In conclusion, it was found that the use of both *rpoB* and *cpn60* genes using either the GenBank or Chaperonin databases provided similar accuracy in the identification of NAS species, although the highest proportion of NAS species were correctly identified with *rpoB* sequencing using the GenBank database. All combinations were unable to identify *S. agnetis* and *S. vitulinus* correctly and there were limitations with the Chaperonin database to identify *S. devriesei.* Although the use of the *rpoB* gene did result in a higher proportion of correctly identified sequences, further identification using *cpn60* could increase the number of sequences available in reference databases and therefore improve the ability to analyze NAS species using this target gene.

Table 2.1. Distribution of 441 non-*aureus* staphylococcal (NAS) sequences, representing 25 species, as categorized among 5 phylogenetic clades

		Number of NAS sequences	
Phylogenetic clade	NAS species	Species	Clade
Clade A	*S. fleuretti*	2	37
	S. sciuri	29	
	S. vitulinus	6	
Clade B	*S. agnetis*	13	99
	S. chromogenes	83	
	S. hyicus	3	
Clade C	*S. simulans*	42	42
Clade D	*S. capitis*	22	122
	S. caprae	1	
	S. devriesei	8	
	S. epidermidis	26	
	S. haemolyticus	29	
	S. hominis	11	
	S. pasteuri	6	
	S. warneri	19	
Clade E	*S. arlettae*	15	141
	S. auricularis	2	
	S. cohnii	24	
	S. equorum	17	
	S. gallinarum	21	
	S. kloosii	1	
	S. nepalensis	2	
	S. saprophyticus	16	
	S. succinus	15	
	S. xylosus	28	
	Total	441	441

Table 2.2. Distribution of non-*aureus* staphylococcal (NAS) sequences identified by species groups with the GenBank database using the *rpoB* gene compared with whole-genome sequencing

		Number of NAS sequences		Correct identification				Incorrect identification	
				Species		Clade		Misidentified	Unidentified
Phylogenetic clade	NAS species	Species	Clade	n	%	n	%	n	n
Clade A	*S. fleuretti*	2	36	2	100.00	30	83.33	0	0
	S. sciuri	28		28	100.00			0	0
	S. vitulinus	6		0	0.00			6	0
Clade B	*S. agnetis*	13	99	1	7.69	87	87.88	12	0
	S. chromogenes	83		83	100.00			0	0
	S. hyicus	3		3	100.00			0	0
Clade C	*S. simulans*	42	42	42	100.00	42	100.00	0	0
Clade D	*S. capitis*	22	122	22	100.00	113	92.62	0	0
	S. caprae	1		1	100.00			0	0
	S. devriesei	8		8	100.00			0	0
	S. epidermidis	26		25	96.15			1	0
	S. haemolyticus	29		29	100.00			0	0
	S. hominis	11		11	100.00			0	0
	S. pasteuri	6		6	100.00			0	0
	S. warneri	19		11	57.89			8	0
Clade E	*S. arlettae*	15	141	15	100.00	115	81.56	0	0
	S. auricularis	2		2	100.00			0	0
	S. cohnii	24		1	4.17			23	0
	S. equorum	17		16	94.12			1	0
	S. gallinarum	21		20	95.24			0	1
	S. kloosii	1		1	100.00			0	0
	S. nepalensis	2		1	50.00			1	0
	S. saprophyticus	16		16	100.00			0	0
	S. succinus	15		15	100.00			0	0
	S. xylosus	28		28	100.00			0	0
	Total	440		387	87.95			52	1

Table 2.3. Distribution of non-*aureus* staphylococcal (NAS) sequences identified by species groups with the GenBank database using the *cpn60* gene compared with whole-genome sequencing

		Number of NAS sequences		Correct identification				Incorrect identification	
				Species		Clade		Misidentified	Unidentified
Phylogenetic clade	NAS species	Species	Clade	n	%	n	%	n	n
Clade A	*S. fleuretti*	2	36	2	100.00	30	83.33	0	0
	S. sciuri	28		28	100.00			0	0
	S. vitulinus	6		0	0.00			6	0
Clade B	*S. agnetis*	13	99	0	0.00	73	73.74	13	0
	S. chromogenes	83		70	84.34			0	13
	S. hyicus	3		3	100.00			0	0
Clade C	*S. simulans*	42	42	40	95.24	40	95.24	0	2
Clade D	*S. capitis*	22	122	22	100.00	92	75.41	0	0
	S. caprae	1		1	100.00			0	0
	S. devriesei	8		8	100.00			0	0
	S. epidermidis	26		1	3.85			25	0
	S. haemolyticus	29		26	89.66			0	3
	S. hominis	11		10	90.91			1	0
	S. pasteuri	6		6	100.00			0	0
	S. warneri	19		18	94.74			1	0
Clade E	*S. arlettae*	15	141	15	100.00	137	97.16	0	0
	S. auricularis	2		2	100.00			0	0
	S. cohnii	24		24	100.00			0	0
	S. equorum	17		17	100.00			0	0
	S. gallinarum	21		20	95.24			0	1
	S. kloosii	1		1	100.00			0	0
	S. nepalensis	2		2	100.00			0	0
	S. saprophyticus	16		16	100.00			0	0
	S. succinus	15		15	100.00			0	0
	S. xylosus	28		25	89.29			0	3
	Total	440		372	84.55			46	22

Table 2.4. Distribution of non-*aureus* staphylococcal (NAS) sequences identified by species groups with the Chaperonin database using the *cpn60* gene compared with whole-genome sequencing

		Number of NAS sequences		Correct identification				Incorrect identification	
				Species		Clade		Misidentified	Unidentified
Phylogenetic clade	NAS species	Species	Clade	n	%	n	%	n	n
Clade A	*S. fleuretti*	2	36	2	100.00	30	83.33	0	0
	S. sciuri	28		28	100.00			0	0
	S. vitulinus	6		0	0.00			6	0
Clade B	*S. agnetis*	13	99	0	0.00	73	73.74	13	0
	S. chromogenes	83		70	84.34			0	13
	S. hyicus	3		3	100.00			0	0
Clade C	*S. simulans*	42	42	40	95.24	40	95.24	0	2
Clade D	*S. capitis*	22	122	22	100.00	103	84.43	0	0
	S. caprae	1		0	0.00			1	0
	S. devriesei	8		0	0.00			0	8
	S. epidermidis	26		26	100.00			0	0
	S. haemolyticus	29		26	89.66			0	3
	S. hominis	11		11	100.00			0	0
	S. pasteuri	6		0	0.00			6	0
	S. warneri	19		18	94.74			1	0
Clade E	*S. arlettae*	15	141	15	100.00	137	97.16	0	0
	S. auricularis	2		2	100.00			0	0
	S. cohnii	24		24	100.00			0	0
	S. equorum	17		17	100.00			0	0
	S. gallinarum	21		20	95.24			0	1
	S. kloosii	1		1	100.00			0	0
	S. nepalensis	2		2	100.00			0	0
	S. saprophyticus	16		16	100.00			0	0
	S. succinus	15		15	100.00			0	0
	S. xylosus	28		25	89.29			0	3
	Total	440		383	87.05			27	30

Table 2.5. Sensitivity (Se) and negative predictive value (NPV) of non-*aureus* staphylococcal (NAS) sequences identified with either the GenBank or Chaperonin databases and the *rpoB* or *cpn60* genes

	GenBank database using the *rpoB* gene (95% CI[1])		GenBank database using the *cpn60* gene (95% CI)		Chaperonin database using the *cpn60* gene (95% CI)	
NAS species	Se	NPV	Se	NPV	Se	NPV
S. chromogenes	100.00 (95.65 - 100.00)	100.00 (98.97 - 100.00)	84.34 (74.71 - 91.39)	96.49 (94.07 - 98.12)	84.34 (74.71 - 91.39)	96.49 (94.07 - 98.12)
S. epidermidis	96.15 (80.36 - 99.90)	99.76 (98.66 - 99.99)	3.85 (0.10 - 19.64)	94.31 (91.71 - 96.28)	100.00 (86.77 - 100.00)	100.00 (99.11 - 100.00)
S. haemolyticus	100.00 (88.06 - 100.00)	100.00 (99.11 - 100.00)	89.66 (72.65 - 97.81)	99.28 (97.90 - 99.85)	89.66 (72.65 - 97.81)	99.28 (97.90 - 99.85)
S. sciuri	100.00 (87.66 - 100.00)	100.00 (99.11 - 100.00)	100.00 (87.66 - 100.00)	100.00 (99.11 - 100.00)	100.00 (87.66 - 100.00)	100.00 (99.11 - 100.00)
S. simulans	100.00 (91.59 - 100.00)	100.00 (99.06 - 100.00)	95.24 (83.84 - 99.42)	99.50 (98.21 - 99.94)	95.24 (83.84 - 99.42)	99.50 (98.21 - 99.94)
S. xylosus	100.00 (87.66- 100.00)	100.00 (99.11 - 100.00)	89.29 (71.77 - 97.73)	99.28 (97.90 - 99.85)	89.29 (71.77 - 97.73)	99.28 (97.90 - 99.85)

[1]95% Confidence interval

CHAPTER 3: THE EFFECTS OF NON-*AUREUS* STAPHYLOCOCCI ON DAIRY HERDS IN SASKATCHEWAN, CANADA

As Chapter 2 demonstrates, by selecting an accurate and efficient way of identifying non-*aureus* staphylococcal (NAS) species, results obtained for both research and diagnostic purposes are much more reliable. Although it is important to accurately identify species, it is equally important to determine the effect that each species has on a cow's health and production. This chapter determines the effects of NAS species on udder health and milk yield for cattle with a NAS intramammary infection (IMI). The majority of quarters with a NAS IMI had higher somatic cell count (SCC) than healthy cows and lower SCC than *Staphylococcus aureus*-positive cows. Non-*aureus* staphylococcal diagnosis appeared to have no effect on milk yield.

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manuscript writing. Luby was responsible for experimental design, data collection and intellectual contribution towards manuscript writing.

3.1 INTRODUCTION

Udder health on dairy farms is a very important issue when it comes to herd management. The detrimental effects of even subclinical IMI can have devastating impacts on a producer's bottom line. It is estimated that the cost of a subclinical IMI is approximately 235 to 420 CAD per case based on milk loss alone (Wilson et al., 1997), however it is often difficult to put a true value on the magnitude of these cases (Rollin et al., 2015). In 2017, it was reported that the second most common reason for animal disposal on dairies across the 4 western Canadian provinces was mastitis or high SCC, representing 16% to 19% of all disposals (CanWest DHI, 2018). Due to this reoccurring problem on many dairies, it is important to understand the effects that even minor pathogens, such as non-*aureus* staphylococci (NAS), have on udder health and milk yield.

Somatic cell count is an indicator of mammary inflammation and is commonly used to evaluate udder health on either an individual cow- or herd-level. It has been shown that cows with a NAS IMI tend to have an elevated SCC compared to uninfected cows (Sampimon et al., 2009b). Although these levels are elevated, these IMI are generally not flagged as an issue, as cows with a NAS IMI tend to have a SCC of <200,000 cells/mL (Condas et al., 2017b), which is considered a 'healthy' SCC level (De Vliegher et al., 2003). As NAS are a group containing multiple species, it is important to evaluate each species individually to see if one has more damaging effects on SCC than another.

There are contradictory results in the literature about the effect that NAS IMI can have on milk yield. Studies have shown that animals with a NAS IMI can have higher (Piepers et al., 2013) or similar (Pearson et al., 2013) milk yield than uninfected animals. It has also been shown that cows with a NAS IMI will have a lower overall milk yield throughout their lactation (Timms and Schultz, 1987). Due to these contrasting results from previous research, additional evaluation of the effect of NAS on milk yield can further our understanding of how NAS impacts udder health.

The objective of this study was to determine the effects of NAS species on udder health and milk yield compared with healthy quarters and quarters infected with a major pathogen. It was hypothesized that NAS species as a group would have only minimal detrimental effects on udder health and milk yield compared to healthy quarters and that there would be distinct differences in udder health and milk yield among NAS groups.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Herds and Animal Selection

A cross-sectional study was conducted on lactating Holstein dairy cattle in Saskatchewan, Canada from the time period of July 2012 to April 2014. A convenience sample of 10 commercial dairy herds with a willingness to participate in the study was selected for data collection. Cattle from all herds were housed in freestall facilities and milked in milking parlours. Herds were selected based on their 2-h proximity to the Western College of Veterinary Medicine (Saskatoon, SK, CA). The study was approved by the University of Saskatchewan Animal Research Ethics Board Animal Care Committee prior to the study commencing (Animal Use Protocol #20110077) and all work with animals was performed according to the Canadian Council on Animal Care guidelines (2009).

Sample size was calculated using online epidemiology software (WinEpi: Working in Epidemiology, University of Zaragoza, Zaragoza, ES; http://www.winepi.net). This calculation was based on an estimated 20% prevalence of NAS from non-clinical milk samples, which is consistent with data collected from the Canadian Bovine Mastitis and Milk Quality Research Network cohort study (Reyher et al., 2012a). Using a 95% confidence interval (CI) and an accepted error of 2.5%, an approximate sample size of 1000 cows was required over 10 herds, with a target of 100 cows being sampled per herd. Cows were selected based on their entry time into the parlour on the first herd visit, with the earliest cows being sampled first. When possible, cows that were ≤250 DIM were selected for study enrollment to minimize the probability of dry-off prior to the end of the study period.

3.2.2 Sample Collection

Each herd was sampled on 3 visits at 3-wk intervals, just prior to milking. Quarter milk samples were collected for bacteriology from enrolled cows on all 3 visits. Approximately 50 mL of milk was aseptically collected from quarters using protocols established by the National Mastitis Council (NMC; Hogan et al., 1999). Samples were transported to the laboratory immediately following the sampling visit then stored at -20°C until cultured.

3.2.3 Milk Culture Analysis

3.2.3.1 Bacteriological Analysis

Samples were cultured using protocols established by the NMC (Hogan et al., 1999). In brief, samples were thawed from frozen at room temperature and milk was streaked on blood agar plates (Tryptone Soya Agar with 5% Sheep Blood, Oxoid Company, Nepean, ON, CA) with sterile cotton-tipped applicator sticks. Plates were incubated at 37°C for 24 and 48 h and all colony types present were quantified, with morphology and hemolysis described. Catalase tests, using hydrogen peroxide as the substrate, were performed on colonies to distinguish between staphylococci and streptococci. Coagulase tests were performed on colonies presumptively identified as staphylococci using rabbit plasma (BBL Rabbit Coagulase Plasma, Becton, Dickinson and Company, Franklin Lakes, NJ, US). Plates that had confirmed NAS colonies based on morphology, catalase and coagulase tests were stored at 4°C until DNA extractions were performed. Samples were considered contaminated if there were ≥3 colony types present on 1 plate at 48 h post-incubation.

Intramammary infections were defined based on modified criteria for all colony types (Andersen et al., 2010). A persistent IMI for all colony types based on morphology identification, as described above, was defined as ≥10 colonies present on a plate at 48 h post-incubation on at least 2 consecutive sampling visits. Quarters that had a persistent IMI of 2 different colonies were categorized as ‘mixed culture’. Quarters that did not meet these criteria were categorized as ‘healthy’.

3.2.3.2 Non-*aureus* Staphylococcal Isolate Preparation

Colonies that were previously identified as NAS were restreaked on blood agar plates with sterile disposable inoculating loops and incubated for 24 h at 37°C. Coagulase tests were performed on colonies presumptively identified as staphylococci, as described above. For freezer stock purposes, 1 colony was used to inoculate 5 mL tryptic soy broth (TSB) for 12 h at 37°C. In a 2 mL cryovial, 1 mL of the resultant NAS suspension in TSB was mixed with 1 mL of 100% glycerol then stored at -80°C.

A 5% chelating resin solution (Chelex 100 Resin, Bio-Rad Laboratories, Hercules, CA, US) was made up for DNA extraction, as described by Walsh et al. (1991). One colony was picked off each plate and placed in a microcentrifuge tube containing 100 µL Chelex solution. Tubes were placed in boiling water for 4-5 min then placed in ice to cool. Tubes were centrifuged for 4 min at 12,000 x g. DNA concentrations were measured using a spectrophotometer (NanoDrop Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, US) and samples were diluted with sterile water to a concentration of 50 ng/µL then stored at -20°C.

3.2.3.3 PCR Procedures

Staphylococcus specific *cpn60* universal primers were developed for PCR procedures and based on H279 and H280 (Goh et al., 1996). Modified primers [StaphF (based on H279) and StaphR (based on H280)] were designed by Dr. Janet Hill (Saskatoon, SK, CA): StaphF (5'-CGCCAGGGTTTTCCCAGTCACGACGAAATYGCTGGKGAYGGTACDACWAC-3') and StaphR (5'-AGCGGATAACAATTTCACACAGGACGWCGATCACCRAADCCWGGHGCYT-3'). These were based on full-length *cpn60* sequences retrieved from the Chaperonin database (Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, CA; http://www.cpndb.ca; Hill et al., 2004). A MasterMix solution (AccuStart Taq DNA Polymerase, Quanta Biosciences, Gaithersburg, MD, US) was formulated for samples, a previously identified NAS positive control and a no-template control.

Amplification of the *cpn60* gene was achieved by conventional PCR. The thermal cycling

conditions consisted of an initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 63.2°C for 30 s and extension at 72°C for 45 s. Final extension occurred at 72°C for 1 min. A select number of samples did not amplify with this protocol and a touchdown method was applied. The protocol was identical to the one previously described, however the first 10 cycles of amplification had a touchdown annealing temperature of 69°C and decreased by 1°C each cycle.

PCR products were analyzed by electrophoresis on a 1.5% agarose gel then purified using a commercial kit (EZ-10 Spin Column PCR Products Purification Kit, Biobasic, Amherst, NY, US or QIAquick PCR Purification Kit, Qiagen, Hilden, DE). Product concentrations were measured using a spectrophotometer and samples were diluted with sterile water to a concentration of ≤60 ng/μL.

3.2.3.4 Sequence Analysis

PCR products were mixed with sequencing primers M1340F (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M1348R (5'-AGCGGATAACAATTTCACACAGGA-3'). Mixtures were shipped to Macrogen (Seoul, KR) for EZ-Seq analysis. Results were retrieved electronically and sequences were analyzed using sequence-assembling software (Staden Package Pregap4 and Gap4, Slashdot Media, San Jose, CA, US; http://www.staden.sourceforge.net). Once sequences were finalized, each file was subjected to a FASTA search (Pearson and Lipman, 1988) on the Chaperonin database (Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, CA; http://www.cpndb.ca; Hill et al., 2004). The NAS species in the database with the greatest homology (≥97%) to the submitted sequence was used for NAS identification; otherwise, sequences were categorized as 'unidentified NAS species'. Quarters identified with 2 different NAS species were categorized as 'mixed NAS species'.

3.2.4 Somatic Cell Count Analysis

Milk samples were collected on the first sampling visit to determine SCC from each quarter. Cows that were enrolled in the study were sampled immediately following the sampling

procedure for bacteriology analysis of milk. Approximately 50 mL of milk was aseptically collected from quarters using protocols established by the NMC (Hogan et al., 1999). Samples were preserved with a bronopol tablet and submitted to the Central Milk Testing Lab (Edmonton, AB, CA) for analysis using flow cytometry (Fossomatic FC, Foss Electric A/S, Hillerød, DK). To minimize extreme outliers that can occur when analyzing SCC, values were converted to SCS. As a reference, SCS = 4 is equivalent to SCC = 200,000 cells/mL. This conversion was done using the following equation (Shook, 1993):

$$SCS = \log^2(SCC/100{,}000) + 3$$

3.2.5 Milk Yield and Cow-Level Data

Cow-level data, including parity and DIM, were collected on the day of study enrollment, as both have been shown to have an impact on SCC and milk yield in quarters infected with NAS (Piepers et al., 2013; Supré et al., 2011). Data were retrieved using on-farm dairy management software (DairyCOMP 305, Valley Agricultural Software, Tulare, CA, US). Milk yield was estimated on a cow-level basis based on routine DHI test day production retrieved from herd recording data (CanWest DHI, Central Milk Testing Lab, Edmonton, AB, CA). Test day production was calculated based on the equivalency of a single milking. Milk yield was followed for the remainder of the study cows' lactations; up to a year following study enrollment.

3.2.6 Data Analyses

Statistical analyses of both SCC and milk yield were performed with statistical software (SAS version 9.4, SAS Institute Inc., Cary, NC). Somatic cell count was analyzed using mixed models (PROC MIXED) that included quarter as the experimental unit, herd and cow within herd as random effects and NAS diagnosis, parity and DIM as fixed effects. Quarters with species-specific NAS diagnosis were used for analysis and compared with healthy quarters and quarters identified with *Staphylococcus aureus* (*S. aureus*), which represented a major pathogen.

Milk yield was analyzed using mixed models with repeated measures (PROC MIXED) that included cow as the experimental unit, herd as a random effect, NAS diagnosis, parity and DIM as fixed effects and time as a repeated measure over cow. Diagnosis categorized cows

based on their number of NAS-positive quarters. Cows were categorized as having 1 NAS-positive quarter or ≥2 NAS-positive quarters. These were compared with cows that had only healthy quarters and cows that had ≥1 *S. aureus*-positive quarter, which represented a major pathogen. Only cows with 4 quarters were included in the analysis. The covariance structure with the lowest Aikake's Information Criterion selected for the model was heterogeneous compound symmetry.

For both models, parity was categorized into primiparous and multiparous groups and DIM was categorized into early (≤100 DIM), mid (101-200 DIM) and late (≥201 DIM) lactation groups. The examination of normality and homogeneity of variances and the detection of outliers or influential points was performed using residuals. The level of significance was set at $P \leq 0.05$.

3.3 RESULTS

A total of 922 cows and 3,688 quarters were enrolled in the study. A mean of 92 (range = 75 to 100) cows per herd were enrolled from the 10 study herds. For analyses, a total of 838 cows and 3,312 quarters were included in the study after 376 quarters were removed, as described in Figure 3.1. A mean of 93 (range = 73 to 100) cows per herd were enrolled from the remaining 9 study herds, which included 304 primiparous cows and 534 multiparous cows. The mean DIM among all remaining study cows at time of enrollment was 146.3 d and was 162.5 and 137.1 d for primiparous and multiparous cows, respectively. The distribution of species identified from the remaining quarter milk samples is reported in Table 3.1.

3.3.1 Somatic Cell Count

A total of 836 cows and 3,196 quarters were included in SCC analysis after 116 quarters were removed, as described in Figure 3.2. A mean of 93 (range = 73 to 100) cows per herd remained from the 9 study herds, which included 302 primiparous and 534 multiparous cows. The mean DIM among all remaining study cows at time of enrollment was 146.6 d and was 163.5 and 137.1 d for primiparous and multiparous cows, respectively.

There was an effect of both DIM and parity on SCS ($P < 0.001$). Late-lactation cows had higher SCS (4.25 ± 0.38) than both early- (3.73 ± 0.38; $P < 0.001$) and mid-lactation (3.73 ± 0.38; $P < 0.001$) cows. Multiparous cows had higher SCS than primiparous cows (4.18 ± 0.37 and 3.63 ± 0.38, respectively). There was also an effect of NAS diagnosis on SCS ($P < 0.001$; Figure 3.3). Healthy quarters had lower SCS than quarters infected with *S. aureus*, *S. capitis*, *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans* and *S. xylosus* ($P \leq 0.02$). Quarters infected with *S. aureus* had higher SCS than quarters infected with *S. chromogenes*, *S. haemolyticus*, *S. hyicus*, *S. lentus* and *S. xylosus* ($P \leq 0.05$), and *S. capitis* had higher SCS than quarters infected with *S. chromogenes* and *S. xylosus* ($P \leq 0.05$).

3.3.2 Milk Yield

A total of 617 cows were included in milk yield analysis after 221 cows were removed, as described in Figure 3.4. A mean of 77 (range = 49 to 94) cows per herd remained from the 8 study herds, which included 230 primiparous and 387 multiparous cows. The mean DIM among all remaining study cows at time of enrollment was 129.2 d and was 136.7 and 124.8 d for primiparous and multiparous cows, respectively.

There was an effect of both DIM and parity on milk yield ($P < 0.001$). Early-lactation cows had higher milk yield (39.71 ± 1.22 kg/d) than both mid- (35.83 ± 1.18 kg/d; $P < 0.001$) and late-lactation (29.19 ± 1.17 kg/d; $P < 0.001$) cows. Mid-lactation cows had higher milk yield than late-lactation cows ($P < 0.001$). Multiparous cows had higher milk yield than primiparous cows (36.10 ± 1.19 and 33.73 ± 1.20 kg/d, respectively). There was no effect of NAS diagnosis on test day milk yield ($P = 0.67$; Figure 3.5).

3.4 DISCUSSION

This study was conducted to evaluate the effect of NAS species on both udder health and milk yield. Many studies have explored the effects of NAS as a group; however there is less research on how individual NAS species can affect dairy cattle. Based on the current knowledge regarding NAS, it was hypothesized that as a group, NAS species would have only minimal

detrimental effects on udder health and milk yield compared to healthy quarters and that there would be a distinct difference in udder health and milk yield among NAS groups.

There were 9 NAS species analyzed for SCC analysis that were compared to healthy and *S. aureus*-positive quarters and it was found that the more prevalent NAS species displayed a higher SCC than healthy quarters, which is similar to what has been reported in previous literature. One group reported that quarters infected with *S. chromogenes*, *S. haemolyticus* and *S. xylosus* also had significantly higher SCC than uninfected quarters (Fry et al., 2014). Another study that had a high prevalence of *S. chromogenes* and *S. xylosus* demonstrated that IMI with these 2 NAS species had increased SCC (Supré et al., 2011). However, these authors reported that the 2 NAS species had a similar SCC level to *S. aureus*, which is contrary to what is reported in the current study. In the current study, there were 4 NAS species (*S. capitis*, *S. cohnii*, *S. epidermidis* and *S. simulans*) that did not differ significantly from the SCC levels of *S. aureus*, however, these all had a low reported prevalence. Further research into these species in herds with a larger prevalence may be beneficial to observe their true effects on udder health.

The observed prevalence of *S. chromogenes* was consistent with that from previous studies; whereby this species represents the majority of NAS isolated on-farm. *Staphylococcus chromogenes* has been isolated from teat apices of heifers as young as 11 mo of age (De Vliegher et al., 2003), indicating that this species may cause IMI that can persist throughout an animal's life. It is important to consider that even though the majority of NAS species do not contribute to extremely high SCC levels as major pathogens do, a NAS IMI may persist well into a cow's lactation and have potential ramifications for future udder health. This is an increased issue with heifers, as producers want these animals to begin their first lactation as healthy as possible to ensure that they have a long and profitable future.

Most quarters infected with NAS species had a higher SCC than healthy quarters, although the majority of these NAS-positive quarters had a SCC of <200,000 cells/mL and were still within the 'healthy' SCC level for udder health (De Vliegher et al., 2003). Traditionally, NAS have been considered minor pathogens with little effect on udder health, while pathogens such as *S. aureus* and coliforms are considered as major pathogens, with more detrimental effects

on the udder (Passchyn et al., 2014). It has been speculated that a quarter previously infected with a NAS IMI is potentially protected against IMI caused by major pathogens (Matthews et al., 1991). However, this association may only be evident in specific types of studies, such as challenge studies, as compared with observational studies (Reyher et al., 2012b). More observational studies should be conducted on this subject to evaluate any potential protective effects of NAS in a field setting.

There was no effect of NAS diagnosis on milk yield compared with healthy quarters. This is similar to what is reported in literature. A study evaluating experimentally-induced quarters with *S. chromogenes* indicated that quarters inoculated with NAS had similar milk yield to control quarters (Simojoki et al., 2009). Similar research conducted on experimentally-induced quarters with either *S. chromogenes* or *S. fleuretti* demonstrated that quarters that were induced with NAS tended to have a lower quarter milk yield than uninfected control quarters (Piccart et al., 2015). These results are similar to what is reported in the current study, however, both Simojoki et al. (2009) and Piccart et al. (2015) observed the effect of experimentally-induced NAS IMI instead of naturally-occurring cases. It is interesting that milk yield did not differ between NAS-positive and *S. aureus*-positive cows or between healthy and *S. aureus*-positive cows. It has previously been shown that cows with *S. aureus*-positive quarters had a lower milk yield than uninfected cows (Gröhn et al., 2004) and it was hypothesized that this would be true in the current study. There were, however, few cows that had quarters solely infected with *S. aureus* in the current study, so milk yield evaluation of these cows compared with both NAS-positive and healthy cows, could be skewed.

Much like SCC, the production impact of an IMI does not necessarily come at the time of pathogen infection, but may influence a cow's subsequent lactation. It has been shown that a NAS IMI can decrease an cow's milk yield throughout her lactation by approximately 800 kg compared to uninfected cows (Timms and Schultz, 1987). However, researchers have indicated that cows with a NAS IMI during a lactation produced 0.5 kg/d of milk more than culture-negative cows (Schukken et al., 2009). In heifers specifically, NAS seem to be very persistent and can greatly influence their overall milk yield. One study found that heifers that freshened with a NAS IMI produced approximately 2 kg/d of milk more than uninfected animals (Piepers

et al., 2013). In contrast, research evaluating the effect of NAS diagnosis in heifers over an entire lactation found that heifers with a positive NAS diagnosis within 30 d of calving did not differ in milk yield from uninfected heifers (Paradis et al., 2010). Similarly, work using 19 pairs of twin heifers found that the heifer that freshened with a NAS IMI had a similar milk yield to their uninfected twin during the first 200 DIM (Pearson et al., 2013). For the current study, study cows were not monitored after freshening; therefore the effects of a NAS IMI were not observed throughout the entire lactation. This would have been valuable information to evaluate, as it is clear that milk yield differs among previous research projects and the true effect of a NAS IMI is not clearly defined.

3.5 CONCLUSIONS

In conclusion, it was found that the majority of NAS species had higher SCC than healthy quarters and lower SCC than *S. aureus*-positive quarters. These SCC values were within the range of what is considered a 'healthy' SCC level for udder health. There was no effect of NAS IMI on overall milk yield compared with both healthy quarters and *S. aureus*-positive quarters. To expand on this research, further longitudinal studies should be conducted to observe the long-term effects of specific NAS species on udder health and milk yield.

Table 3.1. Distribution of pathogens from quarter milk samples based on both parity and lactation groups on Saskatchewan dairy herds

	Number (%) of isolates					
		Parity group		Lactation group (DIM)		
Pathogen	Total	Primiparous	Multiparous	≤100	101-200	≥201
Coliform	2 (0.06)	0 (0.00)	2 (0.06)	2 (0.06)	0 (0.00)	0 (0.00)
NAS[1]						
S. capitis	1 (0.03)	0 (0.00)	1 (0.03)	1 (0.03)	0 (0.00)	0 (0.00)
S. chromogenes	52 (1.57)	22 (0.66)	30 (0.91)	26 (0.79)	16 (0.48)	10 (0.30)
S. cohnii	1 (0.03)	0 (0.00)	1 (0.03)	0 (0.00)	1 (0.03)	0 (0.00)
S. epidermidis	7 (0.21)	0 (0.00)	7 (0.21)	2 (0.06)	3 (0.09)	2 (0.06)
S. haemolyticus	8 (0.24)	1 (0.03)	7 (0.21)	0 (0.00)	4 (0.12)	4 (0.12)
S. hyicus	2 (0.06)	1 (0.03)	1 (0.03)	2 (0.06)	0 (0.00)	0 (0.00)
S. lentus	1 (0.03)	0 (0.00)	1 (0.03)	0 (0.00)	1 (0.03)	0 (0.00)
S. simulans	2 (0.06)	2 (0.06)	0 (0.00)	0 (0.00)	2 (0.06)	0 (0.00)
S. xylosus	15 (0.45)	9 (0.27)	6 (0.18)	0 (0.00)	8 (0.24)	7 (0.21)
Mixed NAS species	2 (0.06)	1 (0.03)	1 (0.03)	1 (0.03)	1 (0.03)	0 (0.00)
Unidentified NAS species	65 (1.96)	24 (0.72)	41 (1.24)	20 (0.60)	23 (0.69)	22 (0.66)
S. aureus	11 (0.33)	6 (0.18)	5 (0.15)	4 (0.12)	4 (0.12)	3 (0.09)
Streptococcus species	8 (0.24)	0 (0.00)	8 (0.24)	3 (0.09)	0 (0.00)	5 (0.15)
Other bacteria	16 (0.48)	1 (0.03)	15 (0.45)	7 (0.21)	3 (0.09)	6 (0.18)
Mixed culture	1 (0.03)	1 (0.03)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.03)
Healthy	3,118 (94.14)	1,131 (34.15)	1,987 (59.99)	1,116 (33.70)	1,178 (35.57)	824 (24.88)
Total	3,312 (100.0)	1,199 (36.20)	2,113 (63.80)	1,184 (35.75)	1,244 (37.56)	884 (26.69)

[1]Non-*aureus* staphylococci

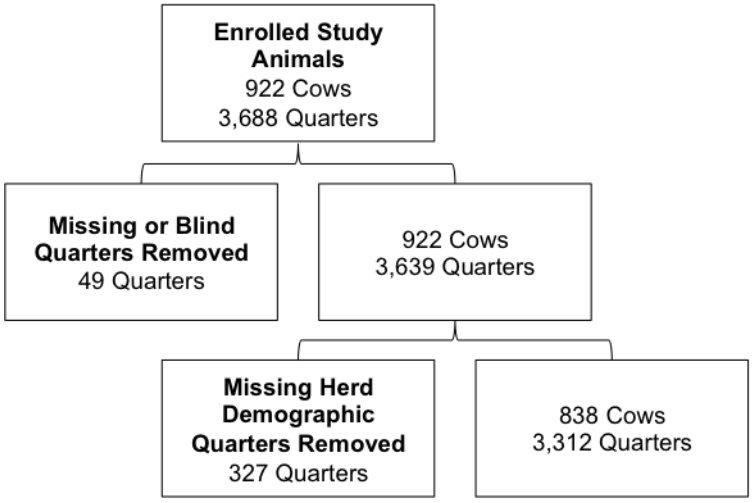

Figure 3.1. Flowchart of quarters and cows included for analyses from an enrolled study group of Saskatchewan dairy herds.

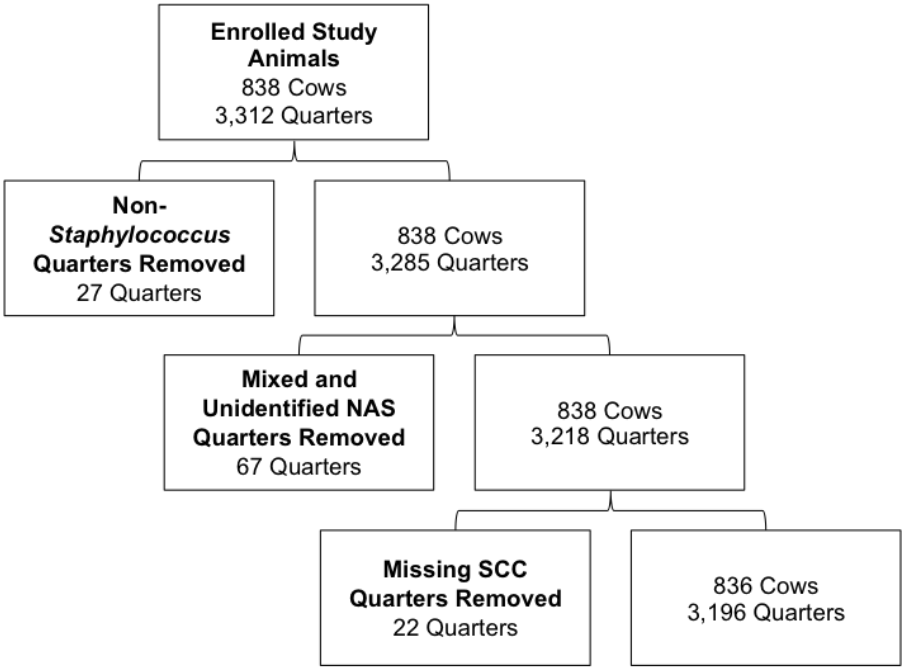

Figure 3.2. Flowchart of quarters and cows included in somatic cell count analysis of Saskatchewan dairy herds.

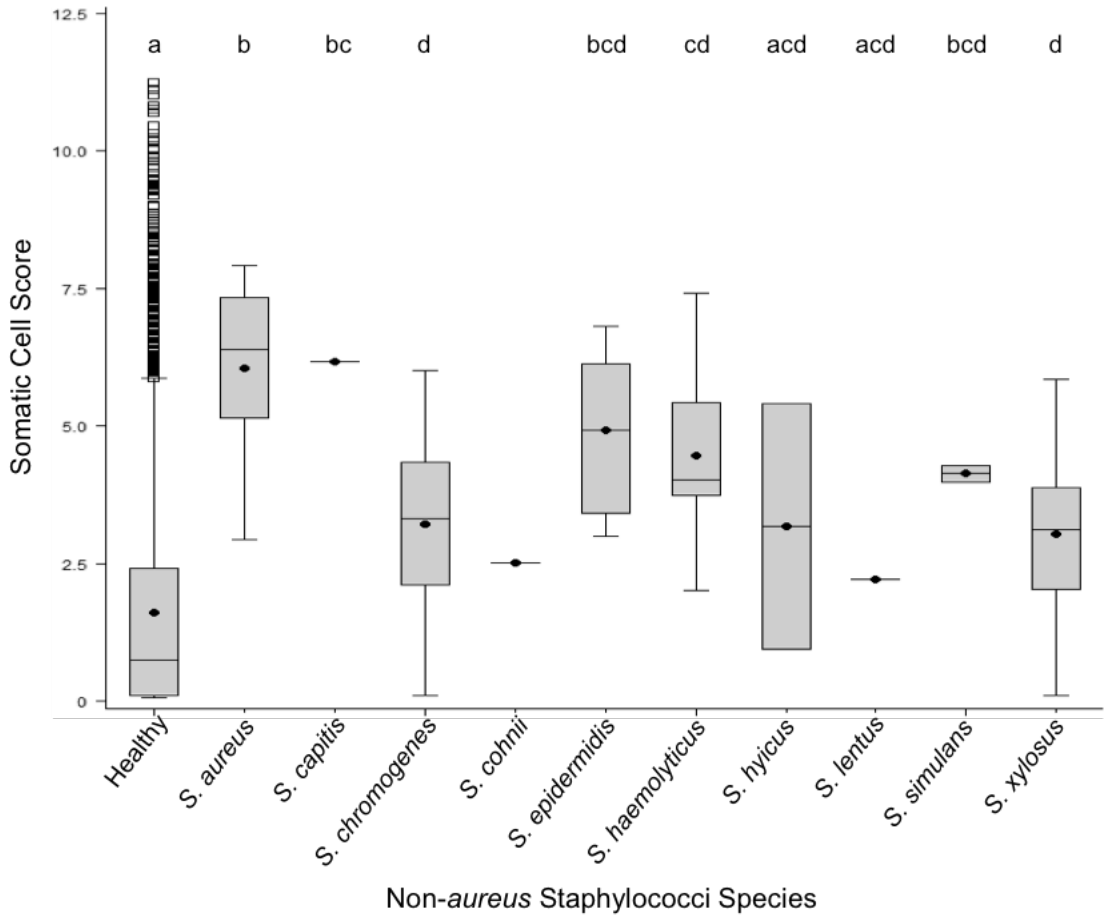

Figure 3.3. Box plot of somatic cell scores of dairy cows from Saskatchewan dairy herds with quarters that were healthy, *Staphylococcus aureus*-positive or non-*aureus* staphylococci-positive.

Each box represents 50% of the data, falling between the 25th and 75th quartile. Horizontal lines within each box represent the median value of the data, black circles represent the mean value of the data and white squares represent outliers. Different letters above each data column indicate significant differences ($P \leq 0.05$).

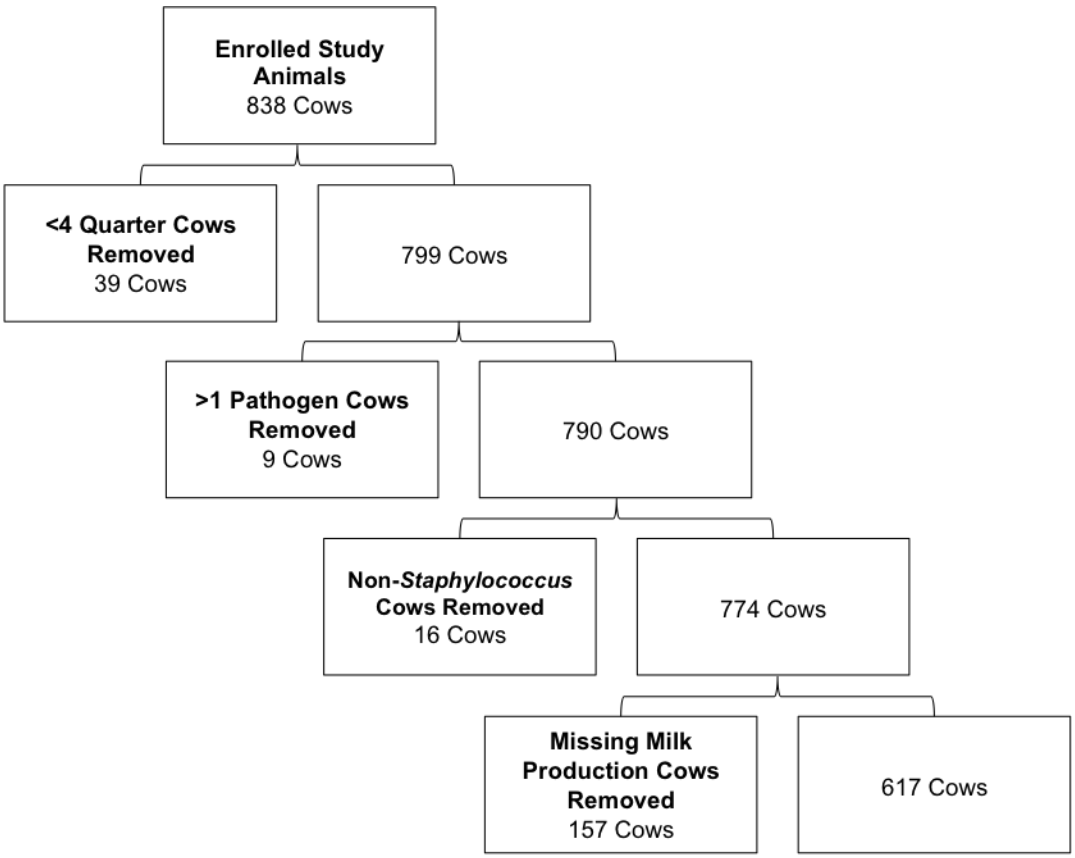

Figure 3.4. Flowchart of cows included in milk yield analysis of Saskatchewan dairy herds.

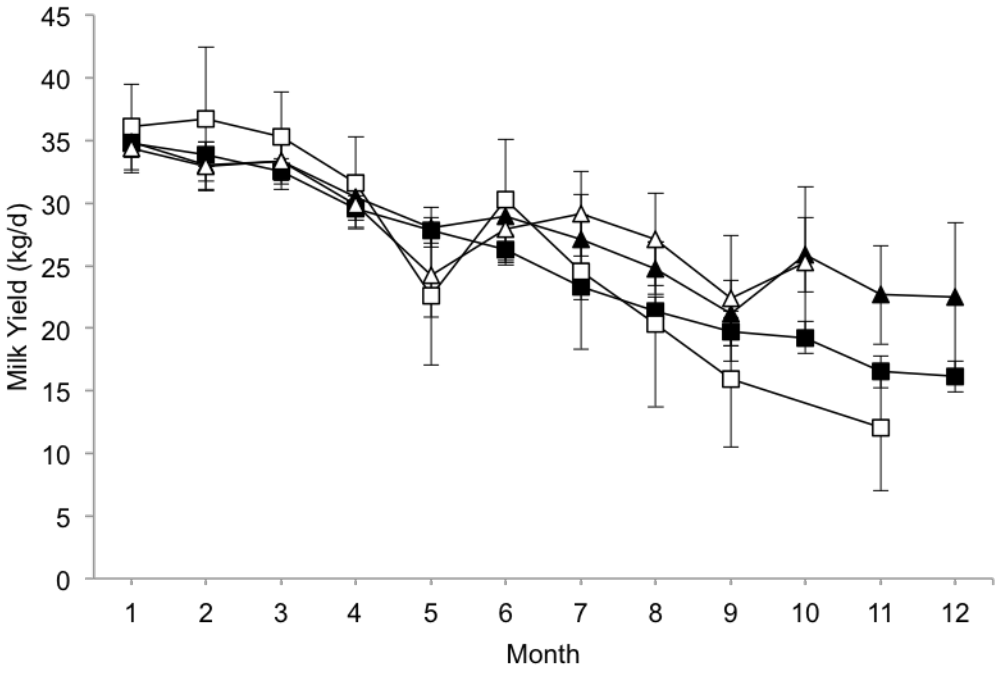

Figure 3.5. Least square mean (±SE) milk yield (kg/d) of dairy cows from Saskatchewan dairy herds that were healthy (■), had *Staphylococcus aureus*-positive quarters (□), had 1 non-*aureus* staphylococci (NAS)-positive quarter (▲) or multiple NAS-positive quarters (△) in the 12 months following study enrollment.

CHAPTER 4: THE COMPARISON OF NON-*AUREUS* STAPHYLOCOCCAL SPECIES ISOLATED FROM MILK SAMPLES WITH THOSE ISOLATED FROM EXTRAMAMMARY SITES TO DETERMINE POTENTIAL SOURCES OF INTRAMAMMARY INFECTIONS

As Chapter 3 demonstrates, increased understanding of how individual non-*aureus* staphylococcal (NAS) species can influence milk yield and somatic cell count on dairy farms makes it important to recognize the source of intramammary infection (IMI). With access to this information, producers can implement control strategies to try to eliminate these IMI. This chapter determines potential sources of NAS IMI and discusses which species exhibit more host-adapted or environmental tendencies. Most frequently, *Staphylococcus chromogenes* (*S. chromogenes*) was found in milk, *S. equorum* was found in both body and environmental sites and *S. xylosus* was found in milk and both extramammary sites. *Staphylococcus xylosus* was the only NAS species that was isolated in the same cow from both milk and extramammary sites.

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Campbell was responsible for intellectual contribution towards data analyses and manuscript writing. White was responsible for intellectual contribution towards laboratory procedures and manuscript writing. Luby was responsible for experimental design, data collection and intellectual contribution towards manuscript writing.

4.1 INTRODUCTION

Prevention strategies to control udder pathogens are key to decreasing IMI. It is important to be aware of the characteristics of each pathogen to have a better understanding of how to control them. Pathogens have traditionally been characterized into 2 categories: environmental and host-adapted (Blowey and Edmondson, 2010). Although there are similar management strategies to decrease IMI between these categories, mainly occurring during teat sanitation prior to and following milking, each group of pathogens have their own unique traits and challenges to address.

Host-adapted pathogens are most commonly transmitted among cows at the time of milking. However, research has demonstrated that it is possible for flies to transmit host-adapted pathogens, such as *Staphylococcus aureus* (*S. aureus*), among cows (Anderson et al., 2012). During milking, there are some key techniques that can be used to minimize pathogen transmission. These include proper sanitation before and after milking, the use of teat dips, ensuring that towels are not shared among cows and milking infected cows last (DFC, 2010). If there are persistent IMI, cows may be selected to be culled from the herd (DFC, 2010).

Environmental pathogens can originate from all housing areas, from both indoor and outdoor facilities. The majority of IMI are caused by pathogens found in manure and generally occur during the dry period or early in lactation (DFC, 2010). Teat dips may help to sanitize and protect teats from environmental pathogens, however, proper manure and housing management are key to decreasing the prevalence of IMI found in a herd (DFC, 2010). Antimicrobial treatment at dry-off and vaccinating for coliform pathogens during the dry period can also decrease existing and prevent new IMI (Jones and Swisher, 2009).

Previous work with non-*aureus* staphylococci (NAS) as a group has characterized them as host-adapted pathogens (Blowey and Edmondson, 2010). However, it is now known that there are many risk factors for a NAS IMI, indicating that these pathogens can exhibit both host-adapted and environmental characteristics (Dufour et al., 2012). It is important to identify where

each individual NAS species originates on-farm so IMI can be prevented and controlled effectively.

The objective of this study was to characterize the distribution of NAS isolated from the environment, body sites and IMI on dairy facilities and to determine potential sources of NAS IMI by comparing isolates from milk samples with those from extramammary sites. It was hypothesized that there would be a within-cow or within-herd agreement for species that have either host-adapted or environmental behaviours.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Herds and Animal Selection

A cross-sectional study was conducted on lactating Holstein dairy cattle in Saskatchewan, Canada from the time period of July 2012 to April 2014. A convenience sample of 10 commercial dairy herds with a willingness to participate in the study was selected for data collection. Cattle from all herds were housed in freestall facilities and milked in milking parlours. Herds were selected based on their 2-h proximity to the Western College of Veterinary Medicine (Saskatoon, SK, CA). The study was approved by the University of Saskatchewan Animal Research Ethics Board Animal Care Committee prior to the study commencing (Animal Use Protocol #20110077) and all work with animals was performed according to the Canadian Council on Animal Care guidelines (2009).

Sample size was calculated using online epidemiology software (WinEpi: Working in Epidemiology, University of Zaragoza, Zaragoza, ES; http://www.winepi.net). This calculation was based on an estimated 20% prevalence of NAS from non-clinical milk samples, which is consistent with data collected from the Canadian Bovine Mastitis and Milk Quality Research Network cohort study (Reyher et al., 2012a). Using a 95% confidence interval (CI) and an accepted error of 2.5%, an approximate sample size of 1000 cows was required over 10 herds, with a target of 100 cows being sampled per herd. Cows were selected based on their entry time into the parlour on the first herd visit, with the earliest cows being sampled first. When possible,

cows that were ≤250 DIM were selected for study enrollment to minimize the probability of dry-off prior to the end of the study period.

4.2.2 Sample Collection

Each herd was sampled on 3 visits at 3-wk intervals, just prior to milking. Quarter milk samples were collected for bacteriology from enrolled cows on all 3 visits. Approximately 50 mL of milk was aseptically collected from quarters using protocols established by the National Mastitis Council (NMC; Hogan et al., 1999). Samples were transported to the laboratory immediately following the sampling visit then stored at -20°C until cultured.

Body site samples were taken on all study cows at the third sampling period. Three sites were selected, including the teat skin, muzzle and perineum. In barns that contained self-locking headgates in the freestall pens, study cows were held in the headgates for sampling and all body site locations were sampled at this time. In barns that did not contain self-locking headgates, the teat skin and perineum of study cows were sampled when the cows were loose in their pens and the muzzle was sampled during milking. For this sampling technique, 2 cm^2 cotton pads were gas sterilized using ethylene oxide. Sterile surgical gloves were used to hold the sterilized cotton pads when swabbing body sites. After swabbing, the gloves were turned inside out to contain the cotton pads and tied in a knot at the base to prevent contamination. Cotton pads were transported in these sterile gloves to the laboratory immediately following the sampling visit. Cotton pads were transferred into a sterile bag (Whirl-Pak Write-On Bags, Nasco, Fort Atkinson, WI, US), immersed in 1 mL tryptic soy broth (TSB) media and stored at -20°C until cultured.

Environmental site samples were taken at the third sampling period. Samples were collected in the lactating cow housing areas from bedding, feedstuffs and water sources in duplicate, as well as the parlour and non-bovine animals (cats and dogs). A handful of bedding and feedstuff samples were collected using sterile surgical gloves. After sample collection, the gloves were turned inside out to contain the samples and tied in a knot at the base to prevent contamination. Samples were transported to the laboratory immediately following the sampling visit then stored at -20ºC until cultured. Sterile 50 mL containers were used to collect water

samples. Containers were dipped in water sources, filled and the lid was resealed to prevent contamination. Samples were transported to the laboratory immediately following the sampling visit then stored at -20°C until cultured. Parlour samples, including milkers' hands, milking cloths and milking units, as well as non-bovine animals, were collected and stored using sterile 2 cm^2 cotton pads, as described above.

4.2.3 Bacteriological Analyses

4.2.3.1 Analysis of Milk Samples

Samples were cultured using protocols established by the NMC (Hogan et al., 1999). In brief, samples were thawed from frozen at room temperature and milk was streaked on blood agar plates (Tryptone Soya Agar with 5% Sheep Blood, Oxoid Company, Nepean, ON, CA) with sterile cotton-tipped applicator sticks. Plates were incubated at 37°C for 24 and 48 h and all colony types present were quantified, with morphology and hemolysis described. Catalase tests, using hydrogen peroxide as the substrate, were performed on colonies to distinguish between staphylococci and streptococci. Coagulase tests were performed on colonies presumptively identified as staphylococci using rabbit plasma (BBL Rabbit Coagulase Plasma, Becton, Dickinson and Company, Franklin Lakes, NJ, US). Plates that had confirmed NAS colonies based on morphology, catalase and coagulase tests were stored at 4°C until DNA extractions were performed. Samples were considered contaminated if there were ≥3 colony types present on 1 plate at 48 h post-incubation.

Intramammary infections were defined based on modified criteria for all colony types (Andersen et al., 2010). A persistent IMI for all colony types based on morphology identification, as described above, was defined as ≥10 colonies present on a plate at 48 h post-incubation on at least 2 consecutive sampling visits. Quarters that had a persistent IMI of 2 different colonies were categorized as 'mixed culture'. Quarters that did not meet these criteria were categorized as 'healthy'.

4.2.3.2 Analysis of Body Site, Parlour and Non-Bovine Animal Samples

Cotton pads were thawed from frozen at room temperature and incubated in 5 mL TSB media for 4 h at 37ºC. A 40 µL aliquot of media was plated and streaked on mannitol salt agar (Chapman Medium, Oxoid Company, Nepean, ON, CA). Plates were incubated at 37ºC and colony types present were quantified, with morphology and hemolysis described, following 24 and 48 h of incubation. Coagulase tests were performed on colonies presumptively identified as staphylococci, as described above. Plates that had confirmed NAS colonies based on morphology and coagulase tests were stored at 4ºC until DNA extractions were performed.

4.2.3.3 Analysis of Housing Area Samples

Housing area samples were thawed from frozen at room temperature. A 5 g sample of bedding and feedstuffs were incubated in 50 mL TSB media for 4 h at 37ºC. A 40 µL aliquot of media was plated on mannitol salt agar. A sample of 10 µL of water was plated and streaked directly on mannitol salt agar. Plates were incubated at 37ºC and colony types present were quantified, with morphology and hemolysis described, following 24 and 48 h of incubation. Coagulase tests were performed on colonies presumptively identified as staphylococci, as described above. Plates that had confirmed NAS colonies were stored at 4ºC until DNA extractions were performed.

4.2.4 Non-*aureus* Staphylococcal Isolate Analysis

4.2.4.1 Isolate Preparation

For all sample types, colonies that were previously identified as NAS were restreaked on blood agar plates with sterile disposable inoculating loops and incubated for 24 h at 37°C. Coagulase tests were performed on colonies presumptively identified as staphylococci, as described above. For freezer stock purposes, 1 colony was used to inoculate 5 mL TSB for 12 h at 37°C. In a 2 mL cryovial, 1 mL of the resultant NAS suspension in TSB was mixed with 1 mL of 100% glycerol then stored at -80°C.

A 5% chelating resin solution (Chelex 100 Resin, Bio-Rad Laboratories, Hercules, CA,

US) was made up for DNA extraction, as described by Walsh et al. (1991). One colony was picked off each plate and placed in a microcentrifuge tube containing 100 µL Chelex solution. Tubes were placed in boiling water for 4-5 min then placed in ice to cool. Tubes were centrifuged for 4 min at 12,000 x g. DNA concentrations were measured using a spectrophotometer (NanoDrop Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, US) and samples were diluted with sterile water to a concentration of 50 ng/µL then stored at -20°C.

4.2.4.2 PCR Procedures

Staphylococcus specific *cpn60* universal primers were developed for PCR procedures and based on H279 and H280 (Goh et al., 1996). Modified primers [StaphF (based on H279) and StaphR (based on H280)] were designed by Dr. Janet Hill (Saskatoon, SK, CA): StaphF (5'-CGCCAGGGTTTTCCCAGTCACGACGAAATYGCTGGKGAYGGTACDACWAC-3') and StaphR (5'-AGCGGATAACAATTTCACACAGGACGWCGATCACCRAADCCWGGHGCYT-3'). These were based on full-length *cpn60* sequences retrieved from the Chaperonin database (Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, CA; http://www.cpndb.ca; Hill et al., 2004). A MasterMix solution (AccuStart Taq DNA Polymerase, Quanta Biosciences, Gaithersburg, MD, US) was formulated for samples, a previously identified NAS positive control and a no-template control.

Amplification of the *cpn60* gene was achieved by conventional PCR. The thermal cycling conditions consisted of an initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 63.2°C for 30 s and extension at 72°C for 45 s. Final extension occurred at 72°C for 1 min. A select number of samples did not amplify with this protocol and a touchdown method was applied. The protocol was identical to the one previously described, however the first 10 cycles of amplification had a touchdown annealing temperature of 69°C and decreased by 1°C each cycle.

PCR products were analyzed by electrophoresis on a 1.5% agarose gel then purified using a commercial kit (EZ-10 Spin Column PCR Products Purification Kit, Biobasic, Amherst, NY,

US or QIAquick PCR Purification Kit, Qiagen, Hilden, DE). Product concentrations were measured using a spectrophotometer and samples were diluted with sterile water to a concentration of ≤60 ng/μL.

4.2.4.3 Sequence Analysis

PCR products were mixed with sequencing primers M1340F (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M1348R (5'-AGCGGATAACAATTTCACACAGGA-3'). Mixtures were shipped to Macrogen (Seoul, KR) for EZ-Seq analysis. Results were retrieved electronically and sequences were analyzed using sequence-assembling software (Staden Package Pregap4 and Gap4, Slashdot Media, San Jose, CA, US; http://www.staden.sourceforge.net). Once sequences were finalized, each file was subjected to a FASTA search (Pearson and Lipman, 1988) on the Chaperonin database (Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, CA; http://www.cpndb.ca; Hill et al., 2004). The NAS species in the database with the greatest homology (≥97%) to the submitted sequence was used for NAS identification; otherwise, sequences were categorized as 'unidentified NAS species'. Quarters identified with 2 different NAS species were categorized as 'mixed NAS species'.

4.2.5 Inclusion Criteria

For the comparison of NAS species retrieved from milk and body site samples, cows were included in the analysis if they were body site sampled on the final sampling visit and had a confirmed NAS IMI. This was indicated with ≥10 NAS colonies present on a plate at 48 h post-incubation on at least 2 consecutive sampling visits and ≥97% homology with a reference database for NAS identification. For the comparison of NAS species retrieved from milk and environmental site samples, cows were included in the analysis if their environment was sampled on the final sampling visit and they had a confirmed NAS IMI, as indicated above.

4.2.6 Data Analyses

Statistical analysis of the association between milk and extramammary sites were

performed with statistical software (SAS version 9.4, SAS Institute Inc., Cary, NC). The association between cows with a NAS-positive IMI and their extramammary sites was analyzed using logistic regression (PROC GLIMMIX) that included cow as the experimental unit and herd as a random effect. The outcome variable was defined as the cow having or not having the same NAS species isolated from both milk and extramammary sites.

4.3 RESULTS

A total of 922 cows and 3,688 quarters were enrolled in the study. A mean of 92 (range = 75 to 100) cows per herd were enrolled from the 10 study herds. The distribution of species cultured from the remaining quarter milk samples is reported in Table 4.1, with 922 cows and 3,639 quarters included in the table after 49 missing or blind quarters were removed. For analyses of only NAS-positive quarters, a total of 141 cows and 174 quarters were included in the study after 3,465 quarters were removed, as described in Figure 4.1. A mean of 14 (range = 2 to 27) cows per herd remained from the 10 study herds.

4.3.1 Milk and Body Site Association

A total of 736 cows were body site sampled in the study. A mean of 73 (range = 46 to 92) cows per herd were body site sampled from the 10 study herds. After the inclusion criteria were met, 90 cows remained in the study. The distribution of NAS species identified from the remaining milk and body site samples is reported in Table 4.2. There was only 1 cow that had the same NAS species identified from both milk and body site samples, which was identified as *S. xylosus*. The probability of having a cow with the same NAS species isolated from both milk and body sites was 0.0101 (95% CI = 0.0010-0.0902).

4.3.2 Milk and Environmental Site Association

Environmental site samples were taken on all 10 study herds; therefore, all 141 NAS-positive cows were included in the analysis, with a mean of 14 (range = 2 to 27) cows per herd. The distribution of NAS species identified from the remaining milk and body site samples is reported in Table 4.3. There were 5 cows that had the same NAS species identified from both

milk and environmental site samples, which were all identified from 1 herd as *S. xylosus*. The probability of having a cow with the same NAS species isolated from both milk and environmental sites was 0.0139 (95% CI = 0.0008-0.1908).

4.4 DISCUSSION

This study was conducted to evaluate the distribution of NAS isolated from the environment, body sites and IMI of dairy facilities. Potential sources of NAS IMI were determined by comparing isolates found in milk samples to those found in extramammary sites, both at the cow- and herd-level. It is important to know where each NAS species is colonized to have a better understanding of how to control these IMI. From what is already known about the origins of NAS species, it was hypothesized that there would be a within-cow or within-herd agreement for species that have either host-adapted or environmental behaviours.

For the more prevalent NAS species isolated from this trial, it was found that samples were isolated in a similar pattern to what has been shown previously. It was found that *S. chromogenes* represented a large proportion of NAS isolates in milk (33.9%), but was not present in body or environmental site samples. Previous research has indicated that *S. chromogenes* has udder-adapted characteristics and is highly prevalent in causing IMI (De Vliegher, 2013), which is also apparent in the current study. As *S. chromogenes* has been shown to represent up to 50% of all NAS IMI on-farm, as well as approximately 30% of clinical mastitis IMI caused by NAS (Condas et al., 2017b), it is important to focus on this pathogen in future research to understand the full extent of its effect on udder health.

The NAS species with the highest proportion in the environment was *S. equorum* (8.4%). Although this species represented a high proportion of samples found in the housing area, it was not isolated from milk. This is similar to what has been shown in previous work, where *S. equorum* had the highest proportion of NAS species isolated from environmental site samples and the lowest from milk samples (Piessens et al., 2011). As there was a high proportion of *S. equorum* from environmental site samples, this species may not be an opportunistic pathogen that causes IMI when an cow is vulnerable, such as *S. haemolyticus* (Piessens et al., 2011).

Staphylococcus equorum was also found in high numbers from NAS-positive body site samples in the current study (17.7%), therefore it can be hypothesized that this pathogen may still be present on the body of cows if they are particularly dirty, but is not necessarily commensal (De Visscher et al., 2016b). Therefore, this pathogen may play a lesser role than other NAS species in terms of its ability to cause IMI.

Staphylococcus xylosus was found in milk, body and environmental site samples and was the only species that was present in both milk and extramammary samples from the same animal. This was similar to a recent study that observed the probability of having the same NAS species from milk and body sites, where heifers with a *S. xylosus* IMI had increased odds of having a body site cultured with *S. xylosus* (Adkins et al., 2018b). However, when strain-typed, *S. xylosus* isolates from milk samples did not share the same strain as isolates from body site samples (Adkins et al., 2018b), indicating that this is a more complex association than previously hypothesized. This is why strain-typing can be an important component to consider when evaluating closely related species, such as NAS, to determine if the same pathogen strain is present, or if it is a variation of the same species.

It is important to address the low number of samples that are included in the analyses, especially in terms of environmental site samples. It is well known that freezing can affect results when analyzing milk by culture (Reyher et al., 2011; Watters et al., 2014). It has been shown that increased freezing time can increase the number of NAS isolates that are quantified on culture plates (Schukken et al., 1989). During the current study, milk samples were frozen for varying periods of time, depending on sampling schedules; therefore it is difficult to determine how the freezing process may have affected each sample. Body site samples were collected in a similar manner to a previous study (Adkins et al., 2018b) and although it was apparent that NAS could be isolated using this strategy, the effect of freezing on these samples is unknown.

4.5 CONCLUSIONS

In conclusion, it was found that there were large proportions of *S. chromogenes* found in milk isolates, *S. equorum* found in body and environmental site isolates and *S. xylosus* found in milk, body and environmental site isolates, which is similar to what has been previously reported

in literature. There was a low proportion of cows with NAS IMI that had the same species present from either a body or environmental site sample. Improved techniques in future research may be beneficial to correctly identify more NAS species from extramammary sites on-farm and get a better understanding of the role these pathogens play on udder health.

Table 4.1. Distribution of pathogens from quarter milk samples on Saskatchewan dairy herds

Pathogen	Number (%) of isolates
Coliform	2 (0.05)
NAS[1]	
S. auricularis	1 (0.03)
S. capitis	2 (0.05)
S. chromogenes	59 (1.62)
S. cohnii	1 (0.03)
S. epidermidis	9 (0.25)
S. haemolyticus	9 (0.25)
S. hyicus	2 (0.05)
S. lentus	1 (0.03)
S. simulans	2 (0.05)
S. xylosus	15 (0.41)
Mixed NAS species	2 (0.05)
Unidentified NAS species	71 (1.95)
S. aureus	16 (0.44)
Streptococcus species	8 (0.22)
Other bacteria	17 (0.47)
Mixed culture	1 (0.03)
Healthy	3,421 (94.01)
Total	3,639 (100.0)

[1]Non-*aureus* staphylococci

Table 4.2. Distribution of non-*aureus* staphylococcal (NAS) species from milk and body site samples on Saskatchewan dairy herds

	Number (%) of isolates	
NAS species	Quarter milk sample	Body site sample
S. arlettae	0 (0.00)	2 (2.08)
S. auricularis	1 (1.08)	0 (0.00)
S. capitis	2 (2.15)	0 (0.00)
S. chromogenes	56 (60.22)	0 (0.00)
S. cohnii	1 (1.08)	2 (2.08)
S. epidermidis	6 (6.45)	1 (1.04)
S. equorum	0 (0.00)	17 (17.71)
S. haemolyticus	8 (8.60)	2 (2.08)
S. hyicus	2 (2.15)	0 (0.00)
S. lentus	1 (1.08)	2 (2.08)
S. nepalensis	0 (0.00)	1 (1.04)
S. saprophyticus	0 (0.00)	2 (2.08)
S. sciuri	0 (0.00)	8 (8.33)
S. simulans	2 (2.15)	0 (0.00)
S. vitulinus	0 (0.00)	8 (8.33)
S. xylosus	14 (15.05)	3 (3.13)
Unidentified NAS species	0 (0.00)	48 (50.00)
Total	93 (100.00)	96 (100.0)

Table 4.3. Distribution of non-*aureus* staphylococcal (NAS) species from milk and environmental site samples on Saskatchewan dairy herds

	Number (%) of isolates	
NAS species	Quarter milk sample	Environmental site sample
S. arlettae	0 (0.00)	2 (1.29)
S. auricularis	1 (1.04)	0 (0.00)
S. capitis	2 (2.08)	0 (0.00)
S. chromogenes	56 (58.33)	0 (0.00)
S. cohnii	1 (1.04)	4 (2.58)
S. epidermidis	7 (7.29)	0 (0.00)
S. equorum	0 (0.00)	13 (8.39)
S. haemolyticus	9 (9.38)	0 (0.00)
S. hyicus	2 (2.08)	0 (0.00)
S. lentus	1 (1.04)	0 (0.00)
S. nepalensis	0 (0.00)	0 (0.00)
S. saprophyticus	0 (0.00)	0 (0.00)
S. sciuri	0 (0.00)	2 (1.29)
S. simulans	2 (2.08)	0 (0.00)
S. vitulinus	0 (0.00)	2 (1.29)
S. xylosus	15 (15.63)	3 (1.94)
Unidentified NAS species	0 (0.00)	129 (83.23)
Total	96 (100.00)	155 (100.00)

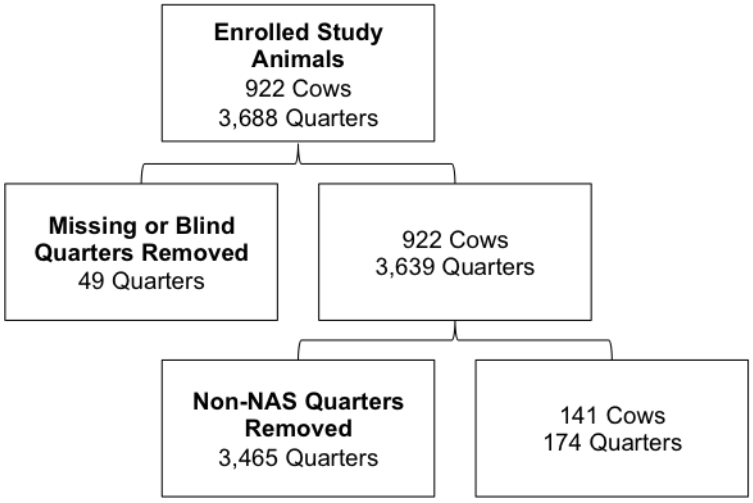

Figure 4.1. Flowchart of non-*aureus* staphylococcal (NAS) quarters and cows included for analyses from an enrolled study group of Saskatchewan dairy herds.

CHAPTER 5: CHARACTERIZTION OF *STAPHYLOCOCCUS CHROMOGENES* GENOTYPES ON DAIRY HERDS ACROSS CANADA

As Chapters 3 and 4 demonstrate, *Staphylococcus chromogenes* (*S. chromogenes*) is the most prevalent non-*aureus* staphylococcal species isolated from intramammary infections (IMI). Exploring the different genotypes of *S. chromogenes* can increase the knowledge of how this species can affect udder health. This chapter evaluates the proportion and persistency of 4 *S. chromogenes* genotypes on dairy farms, as well as the effect that these genotypes have on udder health. Genotype 1 was most predominant and caused the most persistent IMI, having ≥2 samples identified in a quarter throughout a cow's lactation. There was no difference in somatic cell count (SCC) among the genotypes, with all genotypes having a SCC level of <200,000 cells/mL.

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5.1 INTRODUCTION

Non-*aureus* staphylococci (NAS) are the most common pathogens isolated from IMI on dairy farms and of this group, *Staphylococcus chromogenes* (*S. chromogenes*) is the most prevalent (Condas et al., 2017a). A study that identified NAS species and strains isolated from Canadian dairy herds found that *S. chromogenes* accounted for approximately 50% of the NAS isolates (Fry et al., 2014). These authors also found that *S. chromogenes* could cause persistent IMI, with durations between 7 and 224 d. It has been shown that *S. chromogenes* can be isolated from the teat canal approximately 1-2 wk before an IMI is detected in the milk (Quirk et al., 2012), indicating that this NAS species is more complex than previously thought.

There has been contradictory data reported on the effects of *S. chromogenes* on SCC. *Staphylococcus chromogenes* has been associated with an increase in SCC, which is comparable to quarters infected with *S. aureus* (Supré et al., 2011). A recent study observing SCC on Canadian dairy herds demonstrated that *S. chromogenes* isolates had higher SCC than uninfected quarters, however, a higher proportion these isolates had low (<200,000 cells/mL) SCC levels and had lower SCC than major pathogens, including *S. aureus* (Condas et al., 2017b). The contradictory data among studies could be attributed to the differences in *S. chromogenes* at the genotype-level, therefore more research is warranted to observe the effects of genotypes on SCC.

The majority of research conducted on *S. chromogenes* has been done at the species-level. As it has been frequently reported that *S. chromogenes* is the most prevalent NAS species isolated from IMI on dairy farms, it is important to explore this pathogen beyond the species-level to determine if particular genotypes, or strains, have detrimental effects on udder health. Previous studies have reported that as many as 4 different *S. chromogenes* genotypes have been identified on dairy farms (Piessens et al., 2012a). However, this study was conducted on a relatively small number of IMI, therefore it would be beneficial to increase this sample size to get a greater sense of the true impact of each genotype.

The objectives of this study were to determine (1) the proportion of specific *S. chromogenes* genotypes on dairy farms; (2) the effect of these genotypes on SCC; and (3) the persistency of each genotype within quarters. It was hypothesized that there would be a

predominant genotype identified from *S. chromogenes* isolates and that genotypes would yield persistent infections and increased SCC.

5.2 MATERIALS AND METHODS

5.2.1 Sample Selection

Non-*aureus* staphylococcal isolates were acquired from the Canadian Bovine Mastitis and Milk Quality Research Network Mastitis Pathogen Culture Collection (CBMMQRN MPCC; Saint-Hyacinthe QC, CA), as described by Reyher et al. (2011). In brief, 91 herds from the National Cohort of Dairy Farms (NCDF) were enrolled across 6 Canadian provinces (Quebec, Ontario, Alberta, Prince Edward Island, New Brunswick and Nova Scotia) to participate in the project over a 2-yr period between 2007 and 2008.

Milk samples collected on these herds included samples from lactating dairy cows, both with and without clinical mastitis. Study cows were also sampled at calving and dry-off to determine their IMI status throughout their entire lactation. Samples were processed in the laboratory for bacteriological culture using protocols established by the National Mastitis Council (Hogan et al., 1999). Based on these protocols, species that had significant growth on blood agar plates were selected to be stored in the CBMMQRN MPCC (Reyher et al., 2011).

Non-*aureus* staphylococcal isolates were selected from the CBMMQRN MPCC for further species identification. Herd- and cow-level data pertaining to these isolates is described by Condas et al. (2017a).

5.2.2 Non-*aureus* Staphylococcal Isolate Analysis

Identification of NAS species was performed using partial sequencing of the *rpoB* gene, as described by Condas et al. (2017a). In brief, isolates confirmed as NAS were restreaked on blood agar plates and incubated for 24 h at 35°C. DNA extraction was performed, followed by partial sequencing of the *rpoB* gene. Final sequence data were subjected to a BLAST search on the GenBank database (National Center for Biotechnology Information, Bethesda, MD, US;

http://www.ncbi.nlm.nih.gov/genbank; Benson et al., 2013) and compared with reference nucleotide sequences to confirm NAS species identification.

5.2.3 *Staphylococcus chromogenes* Genotype Analysis

The identification of *S. chromogenes* genotypes using high-resolution melt analysis (HRMA) of the 16S rRNA gene was conducted, as described by Ajitkumar et al. (2013). In brief, amplification of 16S rRNA gene fragments from NAS isolates was preformed using real-time PCR. Melt curves and HRMA profiles were analyzed for each isolate and compared with reference sequences. For *S. chromogenes* isolates specifically, 4 genotypes were identified for comparison based on distinctive melt curves.

5.2.4 Data Analyses

Descriptive data analysis was conducted on all 4 *S. chromogenes* genotypes. Both herd- and cow-level data for were retrieved from the CBMMQRN MPCC (Reyher et al., 2011) and the distribution of these characteristics were summarized by each specific genotype.

To minimize extreme outliers that can occur when analyzing SCC, values were converted to SCS. As a reference, SCS = 4 is equivalent to SCC = 200,000 cells/mL. This conversion was done using the following equation (Shook, 1993):

$$SCS = \log^2(SCC/100{,}000) + 3$$

Statistical analyses of SCC and IMI persistency were performed with statistical software (SAS version 9.4, SAS Institute Inc., Cary, NC). Somatic cell count was analyzed using mixed models (PROC MIXED) that included quarter as the experimental unit, cow as a random effect and genotype, parity and DIM as fixed effects. Parity was categorized into primiparous and multiparous groups and DIM was categorized into early (≤100 DIM), mid (101-200 DIM) and late (≥201 DIM) lactation groups. The examination of normality and homogeneity of variances and the detection of outliers or influential points was performed using residuals. The level of significance was set at $P \leq 0.05$.

The persistency of *S. chromogenes* IMI was analyzed using logistic regression (PROC GLIMMIX) that included quarter as the experimental unit and cow as a random effect. The outcome variable was defined as the cow having or not having a persistent IMI. An IMI was defined as a quarter having ≥1 samples from the same lactation identified with the same *S. chromogenes* genotype. A persistent infection was defined as a quarter having ≥2 samples from the same lactation identified with the same *S. chromogenes* genotype.

5.3 RESULTS

5.3.1 Descriptive Data

A total of 2,732 *S. chromogenes* isolates were identified from the CBMMQRN MPCC. These isolates represented all 91 NCDF herds and included 948 cows and 1,253 quarters. For analyses, a total of 2,489 isolates were included in the study after 1 isolate was removed due to missing cow-level data and 242 isolates could not be assigned to 1 of 4 genotypes. The distribution of these isolates among the 4 genotypes was as follows: Genotype 1 [number of samples (n) = 1,342; 53.90%], Genotype 2 (n = 208; 8.35%), Genotype 3 (n = 560; 22.49%) and Genotype 4 (n = 380; 15.26%). The distribution of both cow- and herd-level parameters of the *S. chromogenes* genotypes is described in Table 5.1. There was missing housing type data from 5 isolates and missing SCC level data from 51 isolates, leaving 2,484 and 2,438 isolates, respectively, remaining for descriptive analysis (Table 5.1).

5.3.2 Somatic Cell Count

As indicated above, a total of 2,438 isolates were included in SCC analysis. There was an effect of both DIM and parity on SCS ($P < 0.001$). Mid-lactation cows had lower SCS (3.06 ± 0.10) than both early- (3.44 ± 0.08; $P < 0.001$) and late-lactation (3.48 ± 0.08; $P < 0.001$) cows. Multiparous cows had higher SCS than primiparous cows (3.64 ± 0.08 and 3.02 ± 0.08, respectively). There was no effect of genotype on SCS ($P = 0.76$; Figure 5.1). Both Genotype 3 (3.38 ± 0.09) and Genotype 4 (3.38 ± 0.10) had the highest SCS among the genotypes, followed by Genotype 1 (3.31 ± 0.07) and Genotype 2 (3.24 ± 0.13).

5.3.3 Persistency of Intramammary Infections

A total of 1536 individual IMI were identified from the *S. chromogenes* isolates. Of these IMI, 790 (51.43%) were from Genotype 1, followed by 346 (22.53%) from Genotype 3, 254 (16.54%) from Genotype 4 and 146 (9.51%) from Genotype 2. There were 512 persistent IMI identified, with a mean of 2.86 (range = 2 to 9) isolates per IMI and a mean IMI length of 53.55 (range = 1 to 392) d. On an individual genotype-level, the probability of having a persistent infection for Genotype 1 was 0.3737 [95% confidence interval (CI) = 0.3394-0.4094]; Genotype 2 was 0.2345 (95% CI = 0.1712-0.3124); Genotype 3 was 0.3139 (95% CI = 0.2658-0.3664); and Genotype 4 was 0.2810 (95% CI = 0.2278-0.3411). The distribution of IMI that were identified as persistent or non-persistent for each genotype is reported in Table 5.2.

5.4 DISCUSSION

This study was conducted to evaluate the proportion and persistency of *S. chromogenes* genotypes on dairy farms and to determine the effect of these genotypes on SCC. There has been a vast amount of research conducted on *S. chromogenes* as a species; however, little research has been done on specific genotypes. By expanding this research to the genotype-level, this work has provided an understanding of how *S. chromogenes* affects the udder of dairy cows. It was hypothesized that there would be a predominant genotype identified from *S. chromogenes* isolates and that genotypes would yield persistent infections and increased SCC, as indicated in previous research at the species-level.

In the current study, 51.4% of all isolates were identified as Genotype 1. This is similar to work using both amplification fragment length polymorphism (AFLP) and random amplified polymorphic DNA analyses that identified 4 individual *S. chromogenes* genotypes; the largest of which having a sample proportion of 65% (Piessens et al., 2012a). Contrary to this, another study using pulsed-field gel electrophoresis (PFGE) analysis to evaluate 76 isolates had a predominant strain with a much lower sample proportion of 22% (Mørk et al., 2012); however, authors identified 17 *S. chromogenes* strains, in comparison to the 4 genotypes identified in the current study. Research evaluating *S. chromogenes* strains among clinical mastitis isolates using PFGE found that among the 8 identified strains, the predominant strain had a sample proportion of 36%

(Taponen et al., 2008). It is evident that the method of identifying *S. chromogenes* genotypes or strains can significantly affect the proportion of isolates within each subtype, so caution should be taken when interpreting results from multiple studies.

Somatic cell counts of quarters infected with *S. chromogenes* have been reported to be higher than uninfected quarters, although quarters with these SCC levels would be considered healthy (Fry et al., 2014). In the current study there was no effect of *S. chromogenes* genotype on SCC and all genotypes had 'healthy' SCC levels of <200,000 cells/mL (De Vliegher, 2013). Evaluating SCC among other NAS species may yield different results among genotypes, such as with *S. simulans*, which has been reported to have higher SCC than *S. chromogenes* (Fry et al., 2014; Condas et al., 2017b). There were 15 *S. simulans* strains identified in previous work (Mørk et al., 2012) compared to the 4 genotypes identified in the current study, therefore there could be differences in SCC among these strains.

At a species-level, *S. chromogenes* has been shown to be a persistent pathogen in the udder (Fry et al., 2014; Mørk et al., 2012; Supré et al., 2011), which is also evident in the current study. Upon further analysis at the genotype-level, it was shown in the current study that 23.3-37.6% of IMI were persistent among all 4 genotypes. Another manuscript that identified 4 genotypes from *S. chromogenes* IMI found that 45-67% of IMI were persistent on a genotype-level, however, only 3 genotypes were shown to cause persistent IMI (Piessens et al., 2012a). The difference in persistency among genotypes from these 2 studies may be partially explained by the sample size of the studies, as Piessens et al. (2012a) used only 17 IMI for analysis, while the current study used 1,536 IMI. In addition, an infection was considered to be persistent in the previous study when 2 consecutive samples had the same genotype identified using AFLP, while an infection was considered to be persistent in the current study when ≥2 samples from the same lactation had the same genotype identified using HRMA. Using PFGE, Mørk et al. (2012) also found that multiple *S. chromogenes* strains caused persistent infections, but only 65% of the identified strains were considered to be persistent. These discrepencies may be due to the vast differences in subtype identification and sample size between the current study and that of Mørk et al. (2012), where the current study identified 4 genotypes among 2,489 isolates, while Mørk et al. (2012) identified 17 strains from 76 isolates.

One limitation of this study was the method of identifying *S. chromogenes* subtypes. High resolution melt analysis is a very quick and efficient method to identify NAS isolates and can yield reproducible results with a high throughput of samples (Tabit, 2016). However, in the current study, HRMA was only able to identify 4 individual *S. chromogenes* genotypes. In contrast, most studies that have evaluated different strains of NAS species from bovine milk have used fingerprinting techniques such as PFGE for strain identification, which have been shown to be highly discriminatory when identifying isolates (Tabit, 2016). These studies have been able to identify a high number of *S. chromogenes* strains from much smaller sample sizes than the current study, ranging from 8 (Taponen et al., 2008) to 33 strains (Gillespie et al., 2009). The use of these techniques in the current study may have altered the number and proportion of *S. chromogenes* subtypes identified and subsequently, the persistency of these subtypes in the udder. Another aspect to consider when evaluating results among studies is the definition of "persistency". In the current study, both the number of samples taken and the duration between sampling visits varied among cows, therefore a more specific definition of "persistency" could not be made. In more recent studies, no 2 definitions of a persistent infection are the same and could include multiple isolates per lactation, multiple lactations per cow or a time restriction between isolated samples (Pate et al., 2012; Gillespie et al., 2009; Fry et al., 2014). Due to these vast differences, caution should be taken when comparing and interpreting persistency results from multiple studies.

5.5 CONCLUSIONS

In conclusion, it was found that Genotype 1 was the most predominant *S. chromogenes* genotype identified from the group of isolates. This genotype also caused the most persistent IMI. Although there was a distinct difference in sample proportion and persistency of *S. chromogenes* genotypes, there was no difference in SCC among the genotypes, with all genotypes having a SCC level of <200,000 cells/mL, which is the benchmark of a healthy quarter. As there is little effect of SCC on genotypes, it may not be necessary to identify *S. chromogenes* past the species-level when evaluating IMI.

Table 5.1. Distribution of *Staphylococcus chromogenes* genotypes based on various study parameters from dairy cows on Canadian dairy herds

	Number (%) of isolates				
Independent variable	Total	Genotype 1	Genotype 2	Genotype 3	Genotype 4
Sample type					
Fresh	191 (7.67)	102 (4.10)	19 (0.76)	34 (1.37)	36 (1.45)
Lactating	1,797 (72.20)	972 (39.05)	142 (5.71)	417 (16.75)	266 (10.69)
Dry	501 (20.13)	267 (10.73)	47 (1.89)	109 (4.38)	78 (3.13)
Quarter					
Left front	667 (26.80)	333 (13.38)	56 (2.25)	160 (6.43)	118 (4.74)
Left rear	594 (23.87)	317 (12.70)	40 (1.61)	148 (5.95)	90 (3.62)
Right front	673 (27.04)	379 (15.23)	62 (2.49)	133 (5.34)	99 (3.98)
Right rear	555 (22.30)	313 (12.58)	50 (2.01)	119 (4.78)	73 (2.93)
Parity					
Primiparous	1,316 (52.87)	693 (27.84)	86 (3.46)	329 (13.22)	208 (8.36)
Multiparous	1,173 (47.13)	648 (26.03)	122 (4.90)	231 (9.28)	172 (6.91)
DIM					
Early lactation[1]	909 (36.52)	489 (19.65)	63 (2.53)	196 (7.87)	161 (6.47)
Mid lactation[2]	511 (20.53)	273 (10.97)	47 (1.89)	107 (4.30)	84 (3.37)
Late lactation[3]	1,069 (42.95)	579 (23.26)	98 (3.94)	257 (10.33)	135 (5.42)
Province					
Alberta	501 (20.13)	286 (11.49)	63 (2.53)	86 (3.46)	66 (2.65)
Atlantic	593 (23.82)	331 (13.30)	80 (3.21)	109 (4.38)	73 (2.93)
Ontario	804 (32.30)	395 (15.87)	39 (1.57)	229 (9.20)	141 (5.66)
Quebec	591 (23.74)	329 (13.22)	26 (1.04)	136 (5.46)	100 (4.02)
Year					
2007	1,676 (67.34)	851 (34.19)	154 (6.19)	427 (17.16)	244 (9.80)
2008	813 (32.66)	490 (19.69)	54 (2.17)	133 (5.34)	136 (5.46)
Season					
Spring	883 (35.48)	481 (19.33)	80 (3.21)	193 (7.75)	129 (5.18)
Summer	1,233 (49.54)	642 (25.79)	99 (3.98)	303 (12.71)	189 (7.59)
Autumn	94 (3.78)	46 (1.85)	10 (0.40)	22 (0.88)	16 (0.64)
Winter	279 (11.21)	172 (6.91)	19 (0.76)	42 (1.69)	46 (1.85)
Housing Type					
Tie-stall	1,351 (54.39)	743 (29.91)	85 (3.42)	360 (14.49)	163 (6.56)
Freestall	879 (35.39)	478 (19.24)	77 (3.10)	156 (6.28)	168 (6.76)
Bedded pack	231 (9.30)	103 (4.15)	45 (1.81)	37 (1.49)	46 (1.85)
Mixed housing	23 (0.93)	14 (0.56)	1 (0.04)	6 (0.24)	2 (0.08)
SCC levels					
Low[4]	1,671 (68.54)	897 (36.79)	140 (5.74)	368 (15.09)	266 (10.91)
High[5]	767 (31.46)	418 (17.15)	62 (2.54)	182 (7.47)	105 (4.31)

[1] ≤100 DIM
[2] 101-200 DIM
[3] ≥201 DIM
[4] <200,000 cells/mL
[5] ≥200,000 cells/mL

Table 5.2. Distribution of *Staphylococcus chromogenes* genotypes that were identified as persistent or non-persistent intramammary infections from dairy cows on Canadian dairy herds

Persistency category	Number (%) of quarters	Mean (range) of samples per infection	Mean (range) of infection length (d)
Genotype 1			
Persistent infection	297 (37.59)	2.86 (2 to 9)	56.22 (4 to 376)
Non-persistent infection	493 (62.41)		
Total	790 (100.00)		
Genotype 2			
Persistent infection	34 (23.29)	2.82 (2 to 6)	53.85 (7 to 392)
Non-persistent infection	112 (76.71)		
Total	146 (100.00)		
Genotype 3			
Persistent infection	109 (31.50)	2.96 (2 to 7)	56.42 (1 to 347)
Non-persistent infection	237 (68.50)		
Total	346 (100.00)		
Genotype 4			
Persistent infection	72 (28.35)	2.75 (2 to 6)	38.08 (7 to 173)
Non-persistent infection	182 (71.65)		

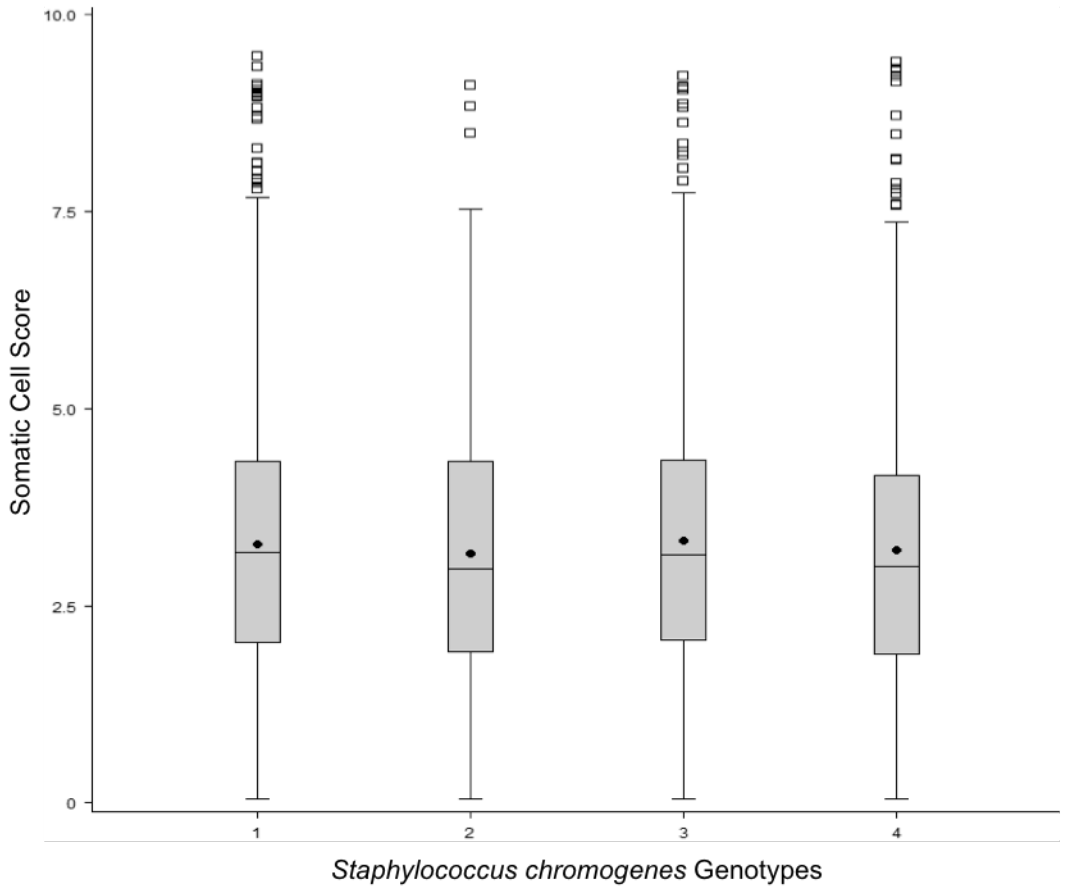

Figure 5.1. Box plot of somatic cell scores of dairy cows on Canadian dairy herds with quarters isolated with *Staphylococcus chromogenes* genotypes.

Each box represents 50% of the data, falling between the 25th and 75th quartile. Horizontal lines within each box represent the median value of the data, black circles represent the mean value of the data and white squares represent outliers.

CHAPTER 6: GENERAL SUMMARY AND CONCLUSIONS

6.1 CONCLUSIONS

The first objective of this series of studies was to evaluate the diagnostic accuracy of different target genes (*rpoB* or *cpn60*) and reference databases (GenBank or Chaperonin) to identify non-*aureus* staphylococcal (NAS) species. It was hypothesized that *cpn60* would be more discriminatory than *rpoB* for identifying NAS species isolated from milk. Non-*aureus* staphylococcal isolates were acquired from milk samples that were confirmed NAS-positive on bacterial culture. Isolates underwent whole-genome sequencing (WGS) and sections of each whole-genome sequence were identified based on 2 target genes (*rpoB* or *cpn60*). Each sequence for both genes was compared with reference sequences from the GenBank database (National Center for Biotechnology Information, Bethesda, MD, US; http://www.ncbi.nlm.nih.gov/genbank; Benson et al., 2013) and *cpn60* sequences were also compared with reference sequences from the Chaperonin database (Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, CA; http://www.cpndb.ca; Hill et al., 2004).

Using the GenBank database, the *rpoB* gene correctly identified 88% of all NAS sequences, followed by the Chaperonin database and *cpn60* gene combination (87.1%) and finally, the GenBank database and *cpn60* gene combination (84.6%). When evaluating the gene and database combinations against WGS results, the GenBank database and *rpoB* gene combination yielded the highest values for specificity and positive predictive value, followed by the Chaperonin database and *cpn60* gene combination and the GenBank database and *cpn60* gene combination. Sensitivity and negative predictive value were 100% for all combinations. Of the incorrectly identified sequences, the *rpoB* gene misidentified the majority of the sequences (98.1%), while the *cpn60* gene had a similar proportion of misidentified and unidentified sequences. This indicates that *cpn60* is highly discriminatory among closely related NAS species, making it a viable option for further identification (Zadoks and Watts, 2009). With all gene and database combinations, there was difficulty identifying both *Staphylococcus agnetis* (*S. agnetis*) and *S. vitulinus*. However, research has shown that these NAS species have been previously misidentified as *S. hyicus* (Calcutt et al., 2014) and *S. pulvereri* (Švec et al., 2004), respectively, so this issue may be due to limitations of reference sequences in both databases.

The results from this study indicate that the GenBank database and *rpoB* gene combination did yield a higher proportion of correctly identified sequences and is an accurate approach for NAS identification. However, by increasing the number of reference sequences available in the Chaperonin database, the *cpn60* gene could be an ideal method for identifying closely related NAS species.

The second objective was to determine the effects of NAS species on udder health and milk yield compared with healthy quarters and quarters infected with a major pathogen. It was hypothesized that NAS species as a group would have only minimal detrimental effects on udder health and milk yield compared to healthy quarters and that there would be distinct differences in udder health and milk yield among NAS groups. Quarter samples from study cows on 10 Saskatchewan dairy herds were aseptically collected on 3 visits at 3-wk intervals and further processed in the laboratory for bacteriological analysis. Milk samples were collected on the first sampling visit to determine SCC at the quarter-level. Milk yield recordings were retrieved from on-farm dairy management software and based on the equivalency of a single milking. Recordings were collected at the cow-level and monitored to the end of the cows' lactations.

A total of 922 cows and 3,688 quarters were enrolled in the study. For SCC analysis, 836 cows and 3,196 quarters were included and for milk yield analysis, 617 cows were included, representing NAS-positive, *S. aureus*-positive and healthy quarters and cows. Late-lactation cows had higher somatic cell score (SCS; 4.3 ± 0.4) than both early- (3.7 ± 0.4; $P < 0.001$) and mid-lactation (3.7 ± 0.4; $P < 0.001$) cows and multiparous cows had higher SCS than primiparous cows (4.2 ± 0.4 and 3.6 ± 0.4, respectively; $P < 0.001$). There was an effect of NAS diagnosis on SCS ($P < 0.001$), whereby healthy quarters had lower SCS than *S. aureus*-positive quarters and the majority of NAS-positive quarters. *Staphylococcus aureus*-positive quarters had higher SCS than the majority of NAS-positive quarters. Early-lactation cows had higher milk yield (39.7 ± 1.2 kg/d) than both mid- (35.8 ± 1.2 kg/d; $P < 0.001$) and late-lactation (29.2 ± 1.2 kg/d; $P < 0.001$) cows and mid-lactation cows had higher milk yield than late-lactation cows ($P < 0.001$). Multiparous cows had higher milk yield than primiparous cows (36.1 ± 1.2 and 33.7 ± 1.2 kg/d, respectively; $P < 0.001$). There was no effect of NAS diagnosis on test day milk yield ($P = 0.67$). The SCC results were similar to those reported in literature, where more prevalent

NAS species had higher SCC levels than healthy quarters (Fry et al., 2014), but were still considered a healthy SCC level of <200,000 cells/mL (Condas et al., 2017b). There was no effect of NAS diagnosis on milk yield; however, there are contrasting reports in literature on how milk yield has been affected by NAS species. Researchers have shown that NAS IMI can cause a decrease (Timms and Schultz, 1987), an increase (Schukken et al., 2009), or cause no effect on milk yield (Pearson et al., 2013) compared with uninfected animals. In addition, the majority of production research that has been done at the species-level has been conducted with experimentally-induced quarters (Simojoki et al., 2009; Piccart et al., 2015) and the effects on-farm are generally unknown. Further longitudinal studies would be beneficial to observe the long-term effects of specific NAS species on milk yield.

The third objective was to characterize the distribution of NAS isolated from the environment, body sites and IMI on dairy facilities and to determine potential sources of NAS IMI by comparing isolates from milk samples with those from extramammary sites. It was hypothesized that there would be a within-cow or within-herd agreement for species that have either host-adapted or environmental behaviours. Quarter samples from study cows on 10 Saskatchewan dairy herds were aseptically collected on 3 visits at 3-wk intervals and further processed in the laboratory for bacteriological analysis. Body and environmental site samples were collected on the third sampling visit and further processed in the laboratory for bacteriological analysis. The association between cows with a NAS-positive IMI and their extramammary sites on a cow-level basis was evaluated.

A total of 922 cows and 3,688 quarters were enrolled in the study, with 141 NAS-positive cows included for analyses. For the analysis of milk and body site association, 90 cows remained. The probability of having a cow with the same NAS species isolated from both milk and body sites was 0.0101, with only 1 cow having the same NAS species (*S. xylosus*) identified from both milk and body site samples. For the analysis of milk and environmental site association, 141 cows remained. The probability of having a cow with the same NAS species isolated from both milk and environmental sites was 0.0139, with 5 cows having the same NAS species (*S. xylosus*) identified from both milk and environmental sites. The most prevalent NAS species identified followed the same trends shown in previous research. Due to its udder-adapted

characteristics (De Vliegher, 2013), *S. chromogenes* was identified exclusively from milk samples and represented the largest proportion of isolates, which is consistent with recent studies (Condas et al., 2017b). *Staphylococcus equorum* was identified exclusively from body and environmental site samples, indicating that this pathogen, although abundant, may not be a major cause of IMI (Piessens et al., 2011). *Staphylococcus xylosus* was one of the only NAS species that was identified from all sample types and was isolated from both milk and extramammary sites of an individual cow. This association has also been shown in heifers (Adkins et al., 2018b), therefore it is a NAS species to evaluate in future research.

The fourth set of objectives were to determine (1) the proportion of specific *S. chromogenes* genotypes on dairy farms; (2) the effect of these genotypes on SCC; and (3) the persistency of each genotype within quarters. It was hypothesized that there would be a predominant genotype identified from *S. chromogenes* isolates and that each genotype would yield persistent infections and increased SCC. Non-*aureus* staphylococcal isolates were acquired from milk samples that were confirmed NAS-positive on bacterial culture. Identification of *S. chromogenes* isolates was performed using both partial sequencing of the *rpoB* gene and high-resolution melt analysis (HRMA) of the 16S rRNA gene, which identified 4 separate *S. chromogenes* genotypes. Both herd- and cow-level data, including SCC data, were collected for data analyses.

A total of 2,372 *S. chromogenes* isolates were enrolled in the study, with 2,489 isolates categorized into 4 genotype groups. Genotype 1 was the most predominant group, representing over 50% of the isolates. For SCC analysis, 2,438 isolates were included. Mid-lactation cows had lower SCS (3.1 ± 0.1) than both early- (3.4 ± 0.1; $P < 0.001$) and late-lactation (3.5 ± 0.1; $P < 0.001$) cows. Multiparous cows had higher SCS than primiparous cows (3.6 ± 0.1 and 3.0 ± 0.1, respectively; $P < 0.001$). There was no effect of genotype on SCS ($P = 0.76$). For persistency analysis, 1,536 individual IMI were included. Of these, Genotype 1 represented over 50% of the IMI. There were 512 persistent IMI included. On an individual genotype-level, the probability of having a persistent infection for Genotype 1 was 0.3737; Genotype 2 was 0.2345; Genotype 3 was 0.3139; and Genotype 4 was 0.2810. Genotype 1 was the most predominant, which is consistent with previous research that identified 1 predominant *S. chromogenes* genotype from

study isolates (Piessens et al., 2012a). There was no effect of genotype on SCC and SCC levels associated with all genotypes were within the healthy range. This may not be surprising, as it has been shown that an IMI with *S. chromogenes* has relatively little impact on SCC (Fry et al., 2014). Among genotypes, the current study identified 23-38% of IMI to be persistent. This is lower than a previous study that identified 45-67% of IMI to be persistent among genotypes (Piessens et al., 2012a). Given the vast difference in sample size between the previous and current study (17 vs. 1,536 IMI, respectively), the current study is likely to be more powerful. One limitation when comparing the current study to other research is the method of identifying *S. chromogenes* subtypes. The current study identified 4 genotypes, while other studies have identified upwards of 33 *S. chromogenes* strains (Gillespie et al., 2009), therefore caution should be taken when comparing research.

6.2 PROJECT LIMITATIONS

There were various limitations in this project. From the first study, reported in Chapter 2, the absence of analysis of the 16S rRNA gene compared with WGS does limit the understanding of target genes that are commonly used in udder health research when determining species of NAS. As there are multiple copies of the 16S rRNA gene found in the genome (Větrovský and Baldrian, 2013), determining the correct copy to use would have been difficult and would have limited the accuracy of species identification. However, it would have been interesting to compare the diagnostic efficacy of this target gene with both the *rpoB* and *cpn60* genes.

From the second study, which was reported in Chapter 3, milk yield could not be determined on a quarter-level, as test day production from CanWest DHI is determined based on the equivalency of a single milking at the cow-level. Due to this limitation, the impact of specific NAS species on milk yield could not be evaluated. Although many studies have evaluated the effects of NAS as a group (Tomazi et al., 2015; Pearson et al., 2013), the evaluation of milk yield at the species-level has primarily used experimentally-induced quarters (Simojoki et al., 2009; Piccart et al., 2015). Analysis of naturally-occurring IMI would improve our understanding of how each species interacts with a quarter and how they can affect overall production.

From the third study, which was reported in Chapter 4, there were some limitations with sample size. For milk samples, freezing time may have played a role in these low numbers. It has been shown that increased freezing time can affect the number of NAS colonies identified on culture plates (Schukken et al., 1989). As processing time in the laboratory varied among samples in the current study, this could have had an impact on NAS isolate retrieval. Body site samples were collected in a similar manner to a previous study (Adkins et al., 2018b) and although NAS were isolated using these methods, the effect of freezing on body and environmental site samples is unknown.

From the fourth study, which was reported in Chapter 5, there was a limitation with the method of identifying *S. chromogenes* subgroups. The current study used HRMA to identify *S. chromogenes* genotypes and although this method has been shown to provide quick and efficient identification (Tabit, 2016), only 4 genotypes were identified. Many studies have used fingerprinting techniques, such as pulsed-field gel electrophoresis, to identify *S. chromogenes* subgroups and these studies were able to identify upwards of 33 different strains (Gillespie et al., 2009). Being able to identify an increased number of strains with a large dataset, as used in the current study, would vastly improve our understanding of how IMI from different NAS subtypes can affect udder health.

6.3 FUTURE RESEARCH

There are many avenues that can be explored regarding the role of NAS in udder health. Exploring multiple copies of the 16S rRNA gene identified from a single genome would be extremely useful in evaluating the accuracy of this target gene to identify NAS species. Other studies have evaluated the number of 16S rRNA copies from closely related species (Větrovský and Baldrian, 2013), however it would be beneficial to do this analysis on NAS species isolated from IMI, as 16S rRNA has been a key target gene for many NAS studies.

Although the knowledge obtained from the current study regarding the effects of NAS on milk yield is beneficial, monitoring this parameter to see how quarter-level IMI can affect a cow's entire lactation from the onset of infection would enhance our understanding of the impact

of these pathogens. Large observational studies have evaluated the effects of NAS IMI throughout a lactation; especially focusing on the impact that these pathogens have on heifers (Piepers et al., 2013). Although this previous research has been valuable, it would be interesting to conduct similar studies using individual NAS species at the quarter-level to see if any have a long-term effect on milk yield.

An unrelated research focus could be to explore the ability of NAS to provide protection against major pathogens in the udder. This would require multiple study types, looking at both naturally-occurring and experimentally-induced IMI, as well as a variety of major pathogens. As it has been shown in the current study that NAS species have varying effects on udder health and IMI prevalence, the evaluation of the impacts that individual species have on major pathogens could be beneficial (Reyher et al., 2012b). This research focus could significantly enhance the way NAS IMI are viewed and interpreted.

6.4 IMPLICATIONS

In terms of NAS IMI diagnosis of individual species, this study has demonstrated that the *cpn60* gene can be a useful target gene when identifying NAS species, with results comparable to the *rpoB* gene. This gene has been shown to be highly discriminatory and can efficiently identify closely related species, including NAS. The use of the curated Chaperonin database is the preferred reference database to use with the *cpn60* gene. With the *cpn60* gene and Chaperonin database combination, efficient and accurate identification of NAS species is possible for both research and diagnostic purposes.

The majority of NAS species, including the 2 most prevalent species (*S. chromogenes* and *S. xylosus*), had higher SCC than healthy quarters. Their SCC levels were elevated, but not above what is considered to be a 'healthy' SCC level and were lower than that of *S. aureus*. As *S. chromogenes* was the most predominant species in the study, further research was done at the genotype-level and although 1 genotype proved to be more predominant and persistent than the others, there was no difference in SCC among genotypes. This indicates that the effects of NAS species on udder health may not have to be evaluated past the species-level. *Staphylococcus*

xylosus was the only NAS species identified in both milk and extramammary sites from the same cow. Although beneficial data, observing the association among sample sites at the strain-level would help to determine whether the same NAS strain is present, or if it is a variation of the same species, which could drastically change how the results are interpreted.

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